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(54) Title: PRODUCTION OF HUMANIZED ANTIBODIES IN TRANSGENIC ANIMALS

(57) Abstract: This invention relates to humanized antibodies and antibody preparations produced from transgenic non-human animals. The non-human animals are genetically engineered to contain one or more humanized immunoglobulin loci which are capable of undergoing gene rearrangement and gene conversion in the transgenic non-human animals to produce diversified humanized immunoglobulins. The present invention further relates to novel sequences, recombination vectors and transgenic vectors useful for making these transgenic animals. The humanized antibodies of the present invention have minimal immunogenicity to humans and are appropriate for use in the therapeutic treatment of human subjects.

Production of Humanized Antibodies In Transgenic Animals

5   **Field of the Invention**

This invention relates to humanized antibodies produced from transgenic non-human animals. The non-human animals are genetically engineered to contain one or more humanized immunoglobulin loci which are capable of undergoing gene rearrangement and gene conversion in the transgenic non-human animals to produce

10   diversified humanized immunoglobulins. The present invention further relates to novel sequences, recombination vectors and transgenic vectors useful for making these transgenic animals. The humanized antibodies of the present invention have minimal immunogenicity to humans and are appropriate for use in the therapeutic treatment of human subjects.

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Background of the Invention

The therapy of infectious diseases caused by bacteria, fungi, virus and parasites is largely based on chemotherapy. However, the emergence of drug-resistant organisms requires the continuous development of new antibiotics. Therapies of patients with

20   malignancies and cancer are also based on chemotherapy. However, many of these therapies are ineffective and the mortality of diseased patients is high. For both infectious diseases and cancer, improved and innovative therapies are needed.

Therapy of steroid resistant rejection of transplanted organs requires the use of biological reagents (monoclonal or polyclonal antibody preparations) that reverse the ongoing

25   alloimmune response in the transplant recipient. The major problem of antibody preparations obtained from animals is the intrinsic immunogenicity of non-human immunoglobulins in human patients. In order to reduce the immunogenicity of non-human antibodies, genetic engineering of individual antibody genes in animals has been proposed. In particular, it has been shown that by fusing animal variable (V) region exons

30   with human constant (C) region exons, a chimeric antibody gene can be obtained. However, this approach may only eliminate the immunogenicity caused by the non-human

Fc region, while the remaining non-human Fab sequences may still be immunogenic. In another approach, human immunoglobulin genes for both, heavy and light chain immunoglobulins have been introduced into the genome of mice. While this genetic engineering approach resulted in the expression of human immunoglobulin polypeptides

5   in genetically engineered mice, the level of human immunoglobulin expression is low. This may be due to species-specific regulatory elements in the immunoglobulin loci that are necessary for efficient expression of immunoglobulins. As demonstrated in transfected cell lines, regulatory elements present in human immunoglobulin genes may not function properly in non-human animals.

10   Several regulatory elements in immunoglobulin genes have been described. Of particular importance are enhancers downstream (3') of heavy chain constant regions and intronic enhancers in light chain genes. In addition, other, yet to be identified, control elements may be present in immunoglobulin genes. Studies in mice have shown that the membrane and cytoplasmic tail of the membrane form of immunoglobulin molecules play

15   an important role in expression levels of human-mouse chimeric antibodies in the serum of mice homozygous for the human $C\gamma1$ gene. Therefore, for the expression of heterologous immunoglobulin genes in animals it is desirable to replace sequences that contain enhancer elements and exons encoding transmembrane (M1 exon) and cytoplasmic tail (M2 exon) with sequences that are normally found in the animal in

20   similar positions.

The introduction of human immunoglobulin genes into the genome of mice resulted in expression of a diversified human antibody repertoire in genetically engineered mice. In both mice and humans, antibody diversity is generated by gene rearrangement. This process results in the generation of many different recombined V(D)J segments

25   encoding a large number of antibody molecules with different antigen binding sites. However, in other animals, like rabbits, pigs, cows and birds, antibody diversity is generated by a substantially different mechanism called gene conversion. For example, it is well established that in rabbit and chicken, VDJ rearrangement is very limited (almost 90% of immunoglobulin is generated with the 3'proximal VH1 element) and antibody

30   diversity is generated by gene conversion and hypermutation. In contrast, mouse and

human gene conversion occurs very rarely, if at all. Therefore, it is expected that in animals that diversify antibodies by gene conversion a genetic engineering approach based on gene rearrangement will result in animals with low antibody titers and limited antibody diversity. Thus, the genetic engineering of large animals for the production of non-

5   immunogenic antibody preparations for human therapy requires alternative genetic engineering strategies.

Relevant Literature

The use of polyclonal antibody preparations for the treatment of transplant

10   rejection was recently reviewed by N. Bonnefoy-Berard et al., *J Heart Lung Transplant* 1996; 15(5): 435-442; C. Colby et al., *Ann Pharmacother* 1996; 30(10):1164-1174; M.J. Dugan et al., *Ann Hematol* 1997; 75(1-2):41-46. The use of polyclonal antibody therapies for autoimmune diseases has been described by W. Cendrowski, *Boll Ist Sieroter Milan* 1997; 58(4):339-343; L.K. Kastrukoff et al., *Can J Neurol Sci* 1978; 5(2):175-178; J.E.

15   Walker et al., *J Neurol Sci* 1976; 29(2-4):303-309. The depletion of fat cells using antibody preparations has been described by L. De Clercq et al., *J Anim Sci* 1997; 75(7):1791-1797; J.T. Wright et al., *Obes Res* 1995; 3(3):265-272.

Regulatory elements in immunoglobulin genes have been described by Bradley et al. (1999), *Transcriptional enhancers and the evolution of the IgH locus*; Lauster, R. et

20   al., *Embo J* 12: 4615-23 (1993); Volgina et al., *J Immunol* 165:6400 (2000); Hole et al., *J Immunol* 146:4377 (1991).

Antibody diversification by gene conversion in chicken and rabbit has been described by Bucchini et al., *Nature* 326: 409-11 (1987); Knight et al., *Advances in Immunology* 56: 179-218 (1994); Langman et al., *Res Immunol* 144: 422-46 (1993). The

25   generation of mice expressing human-mouse chimeric antibodies has been described by Pluschke et al., *Journal of Immunological Methods* 215: 27-37 (1998). The generation of mice expressing human-mouse chimeric antibodies with mouse derived membrane and cytoplamic tails has been described by Zou et al., *Science* 262: 1271-1274 (1993); Zou et al. *Curr Biol* 4: 1099-1103. The generation of mice expressing human immunoglobulin

30   polypeptides has been described by Bruggemann et al. *Curr Opin Biotechnol* 8(4): 455-8 (1997); Lonberg et al. *Int Rev Immunol* 13(1):65-93 (1995); Neuberger et al., *Nature* 338:

350-2 (1989). Generation of transgenic mice using a BAC clone has been described by Yang et al., *Nat Biotechnol* 15: 859-65 (1997).

The generation of transgenic rabbits has been described by Fan, J. et al., Pathol Int 49: 583-94 (1999); Brem et al., *Mol Reprod Dev* 44: 56-62 (1996). Nuclear transfer

5   cloning of rabbits has been described by Stice et al., *Biology of Reproduction* 39: 657-664 (1988). Rabbits with impaired immunoglobulin expression have been described by McCartney-Francis et al., *Mol Immunol* 24: 357-64 (1987); Allegrucci, et al., *Eur J Immunol* 21: 411-7 (1991).

The production of transgenic chicken has been described by Etches et al.,

10   *Methods in Molecular Biology* 62: 433-450; Pain et al., *Cells Tissues Organs* 1999; 165(3-4): 212-9; Sang, H., "Transgenic chickens--methods and potential applications", *Trends Biotechnol 12:415* (1994); and in WO 200075300, "Introducing a nucleic acid into an avian genome, useful for transfecting avian blastodermal cells for producing transgenic avian animals with the desired genes, by directly introducing the nucleic acid into the

15   germinal disc of the egg".

Agammaglobulinemic chicken have been described by Frommel et al., *J Immunol* 105(1): 1-6 (1970); Benedict et al., *Adv Exp Med Biol* 1977; 88(2): 197-205.

The cloning of animals from cells has been described by T. Wakayama et al., *Nature* 1998; 394:369-374; J.B. Cibelli et al., *Science* 280:1256-1258 (1998); J.B. Cibelli

20   et al., *Nature Biotechnology* 1998; 16:642-646; A. E. Schnieke et al., *Science* 278: 2130-2133 (1997); K.H. Campbell et al., *Nature* 380: 64-66 (1996).

Production of antibodies from transgenic animals is described in U.S. Patent No. 5,814,318, No. 5,545,807 and No. 5,570,429. Homologous recombination for chimeric mammalian hosts is exemplified in U.S. Patent No. 5,416,260. A method for

25   introducing DNA into an embryo is described in U.S. Patent No. 5,567,607. Maintenance and expansion of embryonic stem cells is described in U.S. Patent No. 5,453,357.

The mechanisms involved in the diversification of the antibody repertoire in pigs, sheep and cows are reviewed in Butler, J. E. (1998), "Immunoglobulin diversity, B-cell and antibody repertoire development in large farm animals", *Rev Sci Tech 17:43*.

30   Antibody diversification in sheep is described in Reynaud, C. A., C. Garcia, W. R. Hein,

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and J. C. Weill (1995), "Hypermutation generating the sheep immunoglobulin repertoire is an antigen-independent process", *Cell 80:115*; and Dufour, V., S. Malinge, and F. Nau. (1996), "The sheep Ig variable region repertoire consists of a single VH family", *J Immunol 156:2163*.

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Summary of the Invention

One embodiment of the present invention provides humanized antibodies (humanized immunoglobulins) having at least a portion of a human immunoglobulin polypeptide sequence.

10   The humanized antibodies of the present invention are made from transgenic non-human animals genetically engineered to contain one or more humanized Ig loci.

Preferably, the humanized antibodies of the present invention are prepared from transgenic non-human animals which generate antibody diversity primarily by gene conversion and hypermutation, e.g., rabbit, pigs, chicken, sheep, cow and horse. The

15   antibodies can be made by immunizing transgenic animals with a desired antigen such as an infectious agent (e.g., bacteria or viruses) or parts or fragments thereof.

Such humanized antibodies have reduced immunogenicity to primates, especially humans, as compared to non-humanized antibodies prepared from non-human animals. Therefore, the humanized antibodies of the present invention are appropriate for

20   use in the therapeutic treatment of human subjects.

Another embodiment of the present invention provides a preparation of humanized antibodies which can be monoclonal antibodies or polyclonal antibodies. Preferred antibody preparations of the present invention are polyclonal antibody preparations which, according to the present invention, have minimal immunogenicity to

25   primates, especially humans.

A preferred preparation of polyclonal antibodies is composed of humanized immunoglobulin molecules having at least a heavy chain or light chain constant region polypeptide sequence encoded by a human constant region gene segment. More preferably, the variable domains of the heavy chains or light chains of the

30   immunoglobulins molecules are also encoded by human gene segments.

In another embodiment, the present invention provides pharmaceutical compositions which include a preparation of humanized antibodies, and a pharmaceutically-acceptable carrier.

Another embodiment of the present invention provides novel sequences from the 5' and 3' flanking regions of the Ig gene segments of non-human animals, preferably, animals which rely primarily on gene conversion in generating the antibody diversity. In particular, the present invention provides novel nucleotide sequences downstream (3', 3-prime) of the genes coding for Cλ in chickens, Cγ and Cκ in rabbits, Cγ1,2,3 in cows and Cγ1,2 in sheep, as well as novel sequences 5' of rabbit Cγ.

In another embodiment, the present invention provides recombination vectors useful for replacing an Ig gene segment of a non-human animal with the corresponding human Ig gene segment. These vectors include a human Ig gene segment which is linked to flanking sequences at the 5' end and the 3' end, wherein the flanking sequences are homologous to the flanking sequences of the target animal Ig gene segment.

Preferred recombination vectors are those useful for the replacement of the animal's Ig constant region. For example, recombination vectors useful for replacing the rabbit heavy chain constant region genes are provided. A preferred vector contains from 5' to 3', a nucleotide sequence as set forth in SEQ ID NO: 12 or SEQ ID NO: 13, or a portion of SEQ ID NO: 12 or SEQ ID NO: 13, a human heavy chain constant region gene segment, a nucleotide sequence as set forth in SEQ ID NO: 10 or a portion of or SEQ ID NO: 10. Another preferred vector contains a nucleotide sequence as set forth in SEQ ID NO: 51, which sequence is characterized as having a human Cγ1 gene linked to flanking sequences from the 5' and 3' flanking regions of a rabbit heavy chain constant region gene.

Recombination vectors are also provided useful for replacing the rabbit light chain constant region genes. A preferred vector contains a nucleotide sequence as set forth in SEQ ID NO: 53, which sequence is characterized as having a human Cκ linked to flanking sequences from the 5' and 3' flanking regions of the rabbit light chain Cκ1 gene.

Other recombination vectors are provided which are useful for replacing the chicken light chain constant region genes. A preferred vector contains a nucleotide

sequence as set forth in SEQ ID NO: 57 which is characterized as having a human Cλ2 linked to flanking sequences from the 5' and 3' flanking regions of the chicken light chain Cλ gene.

Other recombination vectors provided include those useful for replacing the animal's Ig V region elements. For example, a recombination vector useful for replacing a rabbit heavy chain V region element is provided and contains SEQ ID NO: 52. A recombination vector useful for replacing a rabbit light chain V region element is provided and contains SEQ ID NO: 54.

In still another embodiment, the present invention provides transgenic constructs or vectors containing at least one humanized Ig locus, i.e., an Ig locus from a non-human animal or a portion of an Ig locus from a non-human animal wherein the locus or the portion of a locus is genetically modified to contain at least one human Ig gene segment. Such humanized Ig locus has the capacity to undergo gene rearrangement and gene conversion in the non-human animal thereby producing a diversified repertoire of humanized immunoglobulins.

One humanized Ig locus provided by the invention is a humanized heavy chain locus which includes one or more V gene segments, one or more D gene segments, one or more J gene segments, and one or more constant region gene segments, wherein at least one gene segment is a human heavy chain gene segment. The gene segments in the humanized heavy chain locus are juxtaposed with respect to each other in an unrearranged, or partially or fully rearranged configuration. A preferred humanized heavy chain locus contains a human constant region gene segment, preferably, Cα or Cγ. A more preferred humanized locus contains multiple V gene segments and at least one human V gene segment, in addition to a human heavy chain constant region segment. The human V gene segment is placed downstream of the non-human V gene segments.

Another humanized Ig locus is a humanized light chain locus which includes one or more V gene segments, one or more J gene segments, and one or more constant region gene segments, wherein at least one gene segment is a human light chain gene segment. The gene segments in the humanized light chain locus are juxtaposed with respect to each other in an unrearranged or rearranged configuration. A preferred

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humanized light chain locus contains a human constant region gene segment, preferably, Cλ or Cκ. More preferably, the humanized light chain locus further contains multiple V gene segments and at least one human V gene segment. The human V gene segment is placed downstream of the non-human V gene segments. Even more preferably, the

5   humanized light chain locus includes a rearranged human VJ segment, placed downstream of a number of (e.g., 10-100) VL gene segments of either non-human or human origin.

Another embodiment of the present invention is directed to methods of making a transgenic vector containing a humanized Ig locus by isolating an Ig locus or a portion of an Ig locus from a non-human animal, and integrating the desired human Ig gene

10 segment(s) into the isolated animal Ig locus or the isolated portion of an Ig locus. The human Ig gene segment(s) are integrated into the isolated animal Ig locus or the isolated portion of an Ig locus by ligation or homologous recombination in such a way as to retain the capacity of the locus for undergoing effective gene rearrangement and gene conversion in the non-human animal. Integration of a human Ig gene segment by

15 homologous recombination can be accomplished by using the recombination vectors of the present invention.

In another embodiment, the present invention provides methods of making transgenic animals capable of producing humanized antibodies. The transgenic animals can be made by introducing a transgenic vector containing a humanized Ig locus, or a

20 recombination vector containing a human Ig gene segment, into a recipient cell or cells of an animal, and deriving an animal from the genetically modified recipient cell or cells.

Transgenic animals containing one or more humanized Ig loci, and cells derived from such transgenic animals (such as B cells from an immunized transgenic animal) are also provided. The transgenic animals of the present invention are capable of

25 gene rearranging and gene converting the transgenic humanized Ig loci to produce a diversified repertoire of humanized immunoglobulin molecules.

Brief Description of the Drawings

Figure 1. Cow Cγ 3' flanking sequences. Primers are shown in shaded boxes.

30 The 5' primer is in CH3, and the 3' primer is in M1. The sequences of clone 11, clone 3,

and clone 5 are set forth in SEQ ID NO: 3, SEQ ID NO: 4 and SEQ ID NO: 5, respectively.

　　　　Figure 2. Sheep Cγ 3' flanking sequences. Primers are shown in shaded boxes. The 5' primer is in CH3, and the 3' primer is in M2. The sequences of clone 11

5　and clone 1 are set forth in SEQ ID NO: 8 and SEQ ID NO: 9, respectively.

　　　　Figure 3. A novel 3' flanking sequence (SEQ ID NO: 10) of the rabbit Cgamma gene.

　　　　Figure 4. A novel nucleotide sequence (SEQ ID NO: 11) 3' of the rabbit Ckappa 1 gene.

10　　　　Figure 5. Novel nucleotide sequences (SEQ ID NO: 12 and SEQ ID NO: 13) 5' of the rabbit Cgamma gene. The sequences between SEQ ID NO: 12 and SEQ ID NO: 13 (a gap of about 1000 nt) remain to be determined.

　　　　Figure 6. Comparison of human, mouse, rabbit, sheep, cow and camel sequences for the M1 and M2 regions 3' of the Cgamma gene.

15　　　　Figure 7a. DNA construct for the replacement of rabbit Cκ with human Cκ. A 0.5 kb fragment containing a DNA sequence encoding human Ck is flanked by sequences from the rabbit Cκ1 gene. The upstream sequence (5'Cκ) is 2.8 kb, the downstream sequence (3'Cκ) is 2.6 kb. The vector also contains a lox-neo cassette for positive selection and a Hsv-Tk casette for negative selection.

20　　　　Figure 7b. DNA construct for the replacement of rabbit Cγ with human Cγ1. A 1.8 kb fragment containing a DNA sequence encoding human Cγ1 is flanked by sequences from the rabbit Cγ gene. The upstream sequence (5'Cγ) is 1.9 kb, the downstream sequence (3'Cγ) is 3.1 kb. The vector also contains a lox-neo casette for positive selection and a Hsv-Tk cassette for negative selection. The figure is not up to

25　scale.

　　　　Figure 8. DNA fragment (SEQ ID NO: 51) containing a human immunoglobulin heavy chain Cγ1 gene segment flanked by 50 nucleotides derived from the flanking regions of rabbit Cγ gene. Flanking sequences derived from the flanking regions of rabbit Cγ gene are underlined.

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Figure 9. DNA fragment (SEQ ID NO: 52) containing a V gene segment with more than 80% sequence identity with rabbit V elements and encoding a human V element polypeptide sequence. Flanking sequences derived from the flanking regions of rabbit VH1 and J genes are underlined.

5   **Figure 10.** DNA fragment (SEQ ID NO: 53) containing a human immunoglobulin heavy chain Cκ gene segment flanked by 50 nucleotides derived from the rabbit light chain immunoglobulin Kappa1 gene. Flanking sequences derived from the flanking regions of rabbit Cκ gene are underlined.

Figure 11. DNA fragment (SEQ ID NO: 54) containing a V gene segment 10   with more than 80% sequence identity with rabbit V elements and encoding a human V element polypeptide sequence. Flanking sequences derived from the flanking regions of rabbit immunoglobulin V and J genes are underlined.

Figure 12. DNA fragment (SEQ ID NO: 57) containing a gene encoding human immunoglobulin light chain constant region Clambda2 flanked by 50 nucleotides 15   (underlined) derived from the flanking sequences of chicken Clambda gene.

Figure 13. Modification of the chicken light chain locus using the ET system. A chicken genomic BAC clone with the full-length light chain locus was modified by homologous recombination. In a first step Cλ was deleted by insertion of a selection cassette which was in a second homologous recombination step exchanged against the 20   human Cλ gene.

Figure 14. DNA fragment (SEQ ID NO: 58) containing a VJ gene segment with 80% sequence identity with chicken V gene segments and encoding a human VJ immunoglobulin polypeptide. Flanking sequences derived from the flanking regions of chicken immunolgobulin V and J genes are underlined.

25   **Figure 15.** Modified chicken light chain locus.

Detailed Description of the Invention

One embodiment of the present invention provides humanized immunoglobulins (antibodies).

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By "a humanized antibody" or "a humanized immunoglobulin" is meant an immunoglobulin molecule having at least a portion of a human immunoglobulin polypeptide sequence (or a polypeptide sequence encoded by a human Ig gene segment). The humanized immunoglobulin molecules of the present invention can be isolated from a transgenic non-human animal engineered to produce humanized immunoglobulin molecules. Such humanized immunoglobulin molecules are less immunogenic to primates, especially humans, relative to non-humanized immunoglobulin molecules prepared from the animal or prepared from cells derived from the animal.

The term "non-human animals" as used herein includes, but is not limited to, rabbits, pigs, birds (e.g., chickens, turkeys, ducks, geese and the like), sheep, goats, cows and horses. Preferred non-human animals are those animals which rely primarily on gene conversion and/or somatic hypermutation to generate antibody diversity, e.g., rabbit, pigs, birds (e.g., chicken, turkey, duck, goose and the like), sheep, goat, and cow. Particularly preferred non-human animals are rabbit and chicken.

In animals such as human and mouse, there are multiple copies of V, D and J gene segments on the heavy chain locus, and multiple copies of V and J gene segments on a light chain locus. Antibody diversity in these animals is generated primarily by gene rearrangement, i.e., different combinations of gene segments to form rearranged heavy chain variable region and light chain variable region. In other animals (e.g., rabbit, chicken, sheep, goat, and cow), however, gene rearrangement does not play a significant role in the generation of antibody diversity. For example, in rabbit, only a very limited number of the V gene segments, most often the V gene segments at the 3' end of the V-region, are used in gene rearrangement to form a contiguous VDJ segment. In chicken, only one V gene segment (the one adjacent to the D region, or "the 3' proximal V gene segment"), one D segment and one J segment are used in the heavy chain rearrangement; and only one V gene segment (the 3' proximal V segment) and one J segment are used in the light chain rearrangement. Thus, in these animals, there is little diversity among initially rearranged variable region sequences resulting from junctional diversification. Further diversification of the rearranged Ig genes is achieved by gene conversion, a

process in which short sequences derived from the upstream V gene segments replace short sequences within the V gene segment in the rearranged Ig gene.

The term "Ig gene segment" as used herein refers to segments of DNA encoding various portions of an Ig molecule, which are present in the germline of animals

5   and humans, and which are brought together in B cells to form rearranged Ig genes. Thus, Ig gene segments as used herein include V gene segments, D gene segments, J gene segments and C region gene segments.

The term "human Ig gene segment" as used herein includes both naturally occurring sequences of a human Ig gene segment, degenerate forms of naturally occurring

10   sequences of a human Ig gene segment, as well as synthetic sequences that encode a polypeptide sequence substantially identical to the polypeptide encoded by a naturally occurring sequence of a human Ig gene segment. By "substantially" is meant that the degree of amino acid sequence identity is at least about 85%-95%.

A preferred humanized immunoglobulin molecule of the present invention

15   contains at least a portion of a human heavy or light chain constant region polypeptide sequence. A more preferred immunoglobulin molecule contains at least a portion of a human heavy or light chain constant region polypeptide sequence, and at least a portion of a human variable domain polypeptide sequence.

In another embodiment of the present invention, a preparation of humanized

20   antibodies is provided.

By "a preparation of humanized antibodies" or "a humanized antibody preparation" is meant an isolated antibody product or a purified antibody product prepared from a transgenic non-human animal (e.g., serum, milk, or egg yolk of the animal) or from cells derived from a transgenic non-human animal (e.g., a B-cell or a hybridoma cell).

25   A humanized antibody preparation can be a preparation of polyclonal antibodies, which includes a repertoire of humanized immunoglobulin molecules. A humanized antibody preparation can also be a preparation of a monoclonal antibody.

Although the immunogenicity to humans of a humanized monoclonal antibody preparation is also reduced as compared to a non-humanized monoclonal antibody

30   preparation, humanized polyclonal antibody preparations are preferred embodiments of

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the present invention. It has been recognized that humanized monoclonal antibodies still invoke some degree of an immune response (an anti-idiotype response) in primates (e.g., humans) when administered repeatedly in large quantities because of the unique and novel idiotype of the monoclonal antibody. The present inventors have uniquely recognized that

5   the overall immunogenicity of polyclonal antibodies is less dependent on an anti-idiotype response. For example, polyclonal antibodies made from non-human animals with only the constant region elements humanized (e.g., polyclonal antibodies having constant regions encoded by human gene segments, and having variable domains encoded by the endogenous genes of the non-human animal), are substantially non-immunogenic to

10   primates.

        Without intending to be bound to any theory, the present inventors have proposed that the reduced immunogenicity of such a humanized polyclonal antibody preparation is due to the fact that the preparation contains a very large number of different antibodies with many different idiotypes which are to a large extent defined by novel

15   amino acid sequences in the complimentarity determining regions (CDR) of the heavy and light chain. Therefore, upon administration of such preparation into a primate such as a human, the administered amount of each individual immunoglobulin molecule in the preparation may be too low to solicit immune response against each immunoglobulin molecule. Thus, the humanized polyclonal antibody preparation which has many different

20   idiotypes and variable regions has minimal immunogenicity to a recipient, even if the antibodies in the polyclonal antibody preparation are all directed to the same antigen. To further reduce any potential residual immunogenicity, a humanized polyclonal antibody preparation may be prepared which is composed of immunoglobulin molecules having both the variable domains and the constant regions encoded by human Ig gene segments.

25   In a preferred embodiment, the present invention provides an antibody preparation which includes humanized immunoglobulin molecules having at least a portion of a human heavy or light chain constant region polypeptide sequence. More preferably, the humanized immunoglobulines in the antibody preparation of the present invention further contain at least a portion of a human variable domain polypeptide

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sequence, in addition to at least a portion of a human constant region polypeptide
sequence.

Preferred humanized antibody preparations of the present invention are
composed of humanized antibodies made from transgenic non-human animals whose
5   antibody diversity is generated primarily by gene conversion, such as rabbit, birds (e.g.,
chicken, turkey, duck, goose and the like), sheep, goat, and cow; preferably, rabbit and
chicken.

Once a transgenic non-human animal capable of producing diversified
humanized immunoglobulin molecules is made (as further set forth below), humanized
10   immunoglobulins and humanized antibody preparations against an antigen can be readily
obtained by immunizing the animal with the antigen. A variety of antigens can be used to
immunize a transgenic host animal. Such antigens include, microorganism, e.g. viruses
and unicellular organisms (such as bacteria and fungi), alive, attenuated or dead,
fragments of the microorganisms, or antigenic molecules isolated from the
15   microorganisms.

Preferred bacterial antigens for use in immunizing an animal include purified
antigens from *Staphylococcus aureus* such as capsular polysaccharides type 5 and 8,
recombinant versions of virulence factors such as alpha-toxin, adhesin binding proteins,
collagen binding proteins, and fibronectin binding proteins. Preferred bacterial antigens
20   also include an attenuated version of *S. aureus*, *Pseudomonas aeruginosa*, enterococcus,
enterobacter, and *Klebsiella pneumoniae*, or culture supernatant from these bacteria cells.
Other bacterial antigens which can be used in immunization include purified
lipopolysaccharide (LPS), capsular antigens, capsular polysaccharides and/or recombinant
versions of the outer membrane proteins, fibronectin binding proteins, endotoxin, and
25   exotoxin from Pseudomonas aeruginosa, enterococcus, enterobacter, and Klebsiella
pneumoniae.

Preferred antigens for the generation of antibodies against fungi include
attenuated version of fungi or outer membrane proteins thereof, which fungi include, but
are not limited to, Candida albicans, Candida parapsilosis, Candida tropicalis, and
30   Cryptococcus neoformans.

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Preferred antigens for use in immunization in order to generate antibodies against viruses include the envelop proteins and attenuated versions of viruses which include, but are not limited to respiratory synctial virus (RSV) (particularly the F-Protein), Hepatitis C virus (HCV), Hepatits B virus (HBV), cytomegalovirus (CMV), EBV, and

5   HSV.

Therapeutic antibodies can be generated for the treatment of cancer by immunizing transgenic animals with isolated tumor cells or tumor cell lines; tumor-associated antigens which include, but are not limited to, Her-2-neu antigen (antibodies against which are useful for the treatment of breast cancer); CD20, CD22 and CD53

10   antigens (antibodies against which are useful for the treatment of B cell lymphomas), (3) prostate specific membrane antigen (PMSA) (antibodies against which are useful for the treatment of prostate cancer), and 17-1A molecule (antibodies against which are useful for the treatment of colon cancer).

The antigens can be administered to a transgenic host animal in any convenient

15   manner, with or without an adjuvant, and can be administered in accordance with a predetermined schedule.

After immunization, serum or milk from the immunized transgenic animals can be fractionated for the purification of pharmaceutical grade polyclonal antibodies specific for the antigen. In the case of transgenic birds, antibodies can also be made by

20   fractionating egg yolks. A concentrated, purified immunoglobulin fraction may be obtained by chromatography (affinity, ionic exchange, gel filtration, etc.), selective precipitation with salts such as ammonium sulfate, organic solvents such as ethanol, or polymers such as polyethyleneglycol.

For making a monoclonal antibody, spleen cells are isolated from the

25   immunized transgenic animal and used either in cell fusion with transformed cell lines for the production of hybridomas, or cDNAs encoding antibodies are cloned by standard molecular biology techniques and expressed in transfected cells. The procedures for making monoclonal antibodies are well established in the art. See, e.g., European Patent Application 0 583 980 A1 ("Method For Generating Monoclonal Antibodies From

30   Rabbits"), U.S. Patent No. 4,977,081 ("Stable Rabbit-Mouse Hybridomas And Secretion

Products Thereof"), WO 97/16537 ("Stable Chicken B-cell Line And Method of Use
Thereof"), and EP 0 491 057 B1 ("Hybridoma Which Produces Avian Specific
Immunoglobulin G"), the disclosures of which are incorporated herein by reference. In
vitro production of monoclonal antibodies from cloned cDNA molecules has been

5   described by Andris-Widhopf et al., "Methods for the generation of chicken monoclonal
antibody fragments by phage display", *J Immunol Methods 242:159* (2000), and by
Burton, D. R., "Phage display", *Immunotechnology 1:87* (1995), the disclosures of which
are incorporated herein by reference.

     In a further embodiment of the present invention, purified monoclonal or

10   polyclonal antibodies are admixed with an appropriate pharmaceutical carrier suitable for
administration in primates especially humans, to provide pharmaceutical compositions.
Pharmaceutically acceptable carriers which can be employed in the present
pharmaceutical compositions can be any and all solvents, dispersion media, isotonic
agents and the like. Except insofar as any conventional media, agent, diluent or carrier is

15   detrimental to the recipient or to the therapeutic effectiveness of the antibodies contained
therein, its use in the pharmaceutical compositions of the present invention is appropriate.
The carrier can be liquid, semi-solid, e.g. pastes, or solid carriers. Examples of carriers
include oils, water, saline solutions, alcohol, sugar, gel, lipids, liposomes, resins, porous
matrices, binders, fillers, coatings, preservatives and the like, or combinations thereof

20   The present invention is further directed to novel nucleotide sequences and
vectors, as well as the use of the sequences and vectors in making a transgenic non-human
animal which produces humanized immunoglobulins.

     In general, the genetic engineering of a non-human animal involves the
integration of one or more human Ig gene segments into the animal's genome to create

25   one or more humanized Ig loci. It should be recognized that, depending upon the
approach used in the genetic modification, a human Ig gene segment can be integrated at
the endogenous Ig locus of the animal (as a result of targeted insertion, for example), or at
a different locus of the animal. In other words, a humanized Ig locus can reside at the
chromosomal location where the endogenous Ig locus of the animal ordinarily resides, or

30   at a chromosomal location other than where the endogenous Ig locus of the animal

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ordinarily resides. Regardless of the chromosomal location, a humanized Ig locus of the present invention has the capacity to undergo gene rearrangement and gene conversion in the non-human animal thereby producing a diversified repertoire of humanized immunoglobulin molecules. An Ig locus having the capacity to undergo gene

5   rearrangement and gene conversion is also referred to herein as a "functional" Ig locus, and the antibodies with a diversity generated by a functional Ig locus are also referred to herein as "functional" antibodies or a "functional" repertoire of antibodies.

In one embodiment, the present invention provides novel sequences useful for creating a humanized Ig locus and making transgenic animals capable of producing

10   humanized immunoglobulin molecules. In particular, the present invention provides sequences from the 5' and 3' flanking regions of the Ig gene segments of non-human animals, preferably, animals which rely primarily on gene conversion in generating antibody diversity (e.g., rabbit, pigs, sheep, goat, cow, birds such as chicken, turkey, duck, goose, and the like).

15   The 5' and 3' flanking regions of the genes coding for the constant region are particularly important as these sequences contain untranslated regulatory elements (e.g., enhancers) critical for high Ig expression in the serum. The 3' flanking region of the genes coding for the constant region of the heavy chain also contain exons coding for the membranous and cytoplasmic tail of the membrane form of immunoglobulin (Volgina et

20   al. *J Immunol* 165:6400, 2000). It has been previously established that the membrane and cytoplasmic tail of the membrane form of antibodies are critical in achieving a high level of expression of the antibodies in mice sera (Zou et al., *Science* 262:1271, 1993). Thus, the identification of the flanking sequences permits the replacement of exons and intervening introns of the Cγ gene with the human equivalent, and the maintenance of the

25   endogenous exons encoding the transmembrane and cytoplasmic tail regions as well as the endogenous non-coding enhancer sequences.

In one embodiment, the present invention provides 3' flanking sequences of heavy chain constant regions of non-human animals. More particularly, nucleotide sequences downstream (3', 3-prime) of the genes coding for rabbit Cγ, cow Cγ1,2,3, and

30   sheep Cγ1,2 are provided. Especially preferred nucleotide sequences include SEQ ID NO:

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10 (3' of rabbit Cγ), SEQ ID NOS: 3-5 (3' of cow Cγ1,2,3), and SEQ ID NOS: 8-9 (3' of sheep Cγ1,2).

In another embodiment, the present invention provides 3' flanking sequences of light chain constant regions of non-human animals. More particularly, the present

5   invention provides nucleotide sequences downstream (3', 3-prime) of the genes coding for Cκ in rabbits. Especially preferred nucleotide sequences include SEQ ID NO: 11 (3' of rabbit Cκ).

In still another embodiment, the present invention provides 5' flanking sequences of heavy chain constant regions of non-human animals. More particularly,

10   nucleotide sequences upstream (5', 5-prime) of the rabbit Cγ gene are provided. Especially preferred sequences include SEQ ID NO: 12 and SEQ ID NO: 13.

Another embodiment of the present invention provides 5' flanking sequences of light chain constant regions of non-human animals.

Portions of the above novel flanking sequences are provided by the present

15   invention. By "a portion" is meant a fragment of a flanking nucleotide sequence capable of mediating homologous recombination between the human Ig gene segment and the target animal Ig gene segment. Generally, a portion is at least about 200 base pairs, preferably, at least about 400 base pairs, for recombination in animal cells such as ES cells or fibroblasts, and at least about 40 base pairs, preferably at least about 50 base pairs, for

20   recombination in *E. coli*. Examples of portions of the above novel flanking sequences include SEQ ID NOS: 59-60, 61-62, 63-64, 65-66, 67-68 and 69-70 (represented by the underlined sequences in Figures 8-12 and 14, respectively).

In a further aspect, the present invention provides vectors useful for the replacement of an Ig gene segment of a non-human animal with the corresponding human

25   Ig gene segment. These vectors, also referred to herein as "recombination vectors", include a human Ig gene segment which is linked to flanking sequences at the 5' end and the 3' end, wherein the flanking sequences have a degree of homology with the flanking sequences of the target animal Ig gene segment sufficient to mediate homologous recombination between the human gene and the animal gene segments. Generally, at least

30   about 200 bases should be identical between the flanking regions in a recombination

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vector and the flanking regions of the target gene to achieve efficient homologous
recombination in animal cells such as ES cells and fibroblasts; and at least about 40 bases
should be identical to achieve efficient homologous recombination in *E. coli*.

5   Recombination vectors useful for replacing the animal's immunoglobulin
heavy chain constant region genes are provided, which contain from 5' to 3', a nucleotide
sequence homologous to the 5' flanking region of the target animal heavy chain constant
region gene, a human heavy chain constant region gene (e.g., human Cγ1), and a
nucleotide sequence homologous to the 3' flanking region of the target animal heavy
chain constant region gene.

10   Preferred recombination vectors are provided for the replacement of the rabbit
heavy chain constant region genes. One such vector contains from 5' to 3', a nucleotide
sequence as set forth in SEQ ID NO: 12 or SEQ ID NO: 13 or a portion thereof, a human
heavy chain constant region gene segment, a nucleotide sequence as set forth in SEQ ID
NO: 10 or a portion of or SEQ ID NO: 10. Another such vector contains SEQ ID NO: 51
15 (Figure 8) which is characterized as having a human Cγ1 gene linked to flanking
sequences from the 5' and 3' flanking regions of a rabbit heavy chain constant region
gene.

Recombination vectors are also provided which are useful for replacing the
animal's immunoglobulin light chain constant region genes. Such vectors contain from 5'
20 to 3', a nucleotide sequence homologous to the 5' flanking region of the target light chain
constant region gene, a human light chain constant region gene (e.g., human Cκ or Cλ),
and a nucleotide sequence homologous to the 3' flanking region of the target light chain
constant region gene.

Preferred vectors include those useful for replacing the rabbit light chain
25 constant region genes. A preferred vector contains a nucleotide sequence as set forth in
SEQ ID NO: 53, which sequence is characterized as having a human Cκ linked to
flanking sequences from the 5' and 3' flanking regions of the rabbit light chain Cκ1 gene.

Other recombination vectors provided include those useful for replacing the
animal's Ig V region elements. For example, a recombination vector useful for replacing
30 a rabbit heavy chain V region element is provided and contains SEQ ID NO: 52. A

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recombination vector useful for replacing a rabbit light chain V region element is provided and contains SEQ ID NO: 54.

The recombination vectors of the present invention can include additional sequences that facilitate the selection of cells which have undergone a successful recombination event. For example, marker genes coding for resistance to neomycin, bleomycin, puromycin and the like can be included in the recombination vectors to facilitate the selection of cells which have undergone a successful recombination event.

In a further aspect of the present invention, transgenic constructs or vectors carrying one or more humanized Ig loci are provided.

In one embodiment, the present invention provides transgenic constructs containing a humanized Ig heavy chain locus which includes one or more V gene segments, one or more D gene segments, one or more J gene segments, and one or more constant region gene segments, wherein at least one gene segment is a human heavy chain gene segment. The gene segments in such humanized heavy chain locus are juxtaposed wit respect to each other in an unrearranged configuration (or "the germline configuration"), or in a partially or fully rearranged configuration. The humanized heavy chain locus has the capacity to undergo gene rearrangement (if the gene segments are not fully rearranged) and gene conversion in the non-human animal thereby producing a diversified repertoire of heavy chains having human polypeptide sequences, or "humanized heavy chains".

In a preferred embodiment, the humanized heavy chain locus contains at least one C-region gene segment that is a human constant region gene segment, preferably, Cα or Cγ (including any of the Cγ subclasses 1, 2, 3 and 4).

In another more preferred embodiment, the humanized heavy chain locus of the transgene contains a humanized V-region and a humanized C-region, i.e., a V-region having at least one human VH gene segment and a C-region having at least one human C gene segment (e.g., human Cα or Cγ).

Preferably, the humanized V-region includes at least about 10-100 heavy chain V (or "VH") gene segments, at least one of which is a human VH gene segment. In accordance with the present invention, the human VH gene segment included in the

transgene shares at least about 75% to about 85% homology to the VH gene segments of the host animal, particularly those animal VH gene segments included in the upstream region of the transgene. As described above, a human VH segment encompasses naturally occurring sequences of a human VH gene segment, degenerate forms of naturally

5   occurring sequences of a human VH gene segment, as well as synthetic sequences that encode a polypeptide sequence substantially (i.e., at least about 85%-95%) identical to a human heavy chain V domain polypeptide.

Preferably, the human VH gene segment(s) is placed downstream of the non-human VH segments in the transgene locus. Preferably, the non-human VH gene

10   segments in the transgene are the VH gene segments from the 3' VH-region in the Ig locus of the host animal, including the 3' proximal VH1.

In another embodiment, the present invention provides transgenic constructs containing a humanized light chain locus capable of undergoing gene rearrangement and gene conversion in the host animal thereby producing a diversified repertoire of light

15   chains having human polypeptide sequences, or "humanized light chains".

The humanized light locus includes one or more V gene segments, one or more J gene segments, and one or more constant region gene segments, wherein at least one gene segment is a human light chain gene segment. The gene segments in the humanized light chain locus are juxtaposed in an unrearranged configuration (or "the germline

20   configuration"), or fully rearranged configuration.

In a preferred embodiment, the humanized light chain locus contains at least one C-region gene segment that is a human constant region gene segment, preferably, Cλ or Cκ.

In another preferred embodiment, the humanized light chain locus of the

25   transgene contains a humanized V-region and a humanized C-region, e.g., a V-region having at least one human VL gene and/or at least one rearranged human VJ segment, and a C-region having at least one human C gene segment (e.g., human Cλ or Cκ).

Preferably, the humanized V-region includes at least about 10-100 light chain V (or "VL") gene segments, at least one of which is a human VL gene segment. The

30   human VL gene segment included in the transgene shares at least about 75% to about 85%

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homology to the VL gene segments of the host animal, particularly those animal VL gene segments included in the upstream region of the transgene. Consistently, a human VL segment encompasses naturally occurring sequences of a human VL gene segment, degenerate forms of naturally occurring sequences of a human VL gene segment, as well

5   as synthetic sequences that encode a polypeptide sequence substantially (i.e., at least about 85%-95%) identical to a human light chain V domain polypeptide.

Preferably, the human VL gene segment(s) is placed downstream of the non-human VL segments in the transgene locus. The non-human VL gene segments in the transgene construct are selected from the VL gene segments in the 3'VL-region in the

10   light chain locus of the host animal, including the 3' proximal VL1.

In still another preferred embodiment, the humanized light chain locus includes a rearranged human VJ segment, placed downstream of a number of (e.g., 10-100) VL gene segments of either non-human or human origin.

Another aspect of the present invention is directed to methods of making a

15   transgenic vector containing a humanized Ig locus. Such methods involve isolating an Ig locus or a portion thereof from a non-human animal, and inserting the desired human Ig gene segment(s) into the isolated animal Ig locus or the isolated portion of an animal Ig locus. The human Ig gene segment(s) are inserted into the isolated animal Ig locus or a portion thereof by ligation or homologous recombination in such a way as to retain the

20   capacity of the locus of undergoing effective gene rearrangement and gene conversion in the non-human animal.

Preferably, DNA fragments containing an Ig locus to be humanized are isolated from animals which generate antibody diversity by gene conversion, e.g., rabbit and chicken. Such large DNA fragments can be isolated by screening a library of

25   plasmids, cosmids, YACs or BACs, and the like, prepared from the genomic DNA of the non-human animal. An entire animal C-region can be contained in one plasmid or cosmid clone which is subsequently subjected to humanization. YAC clones can carry DNA fragments of up to 2 megabases, thus an entire animal heavy chain locus or a large portion thereof can be isolated in one YAC clone, or reconstructed to be contained in one YAC

30   clone. BAC clones are capable of carrying DNA fragments of smaller sizes (about 150-

250 kb). However, multiple BAC clones containing overlapping fragments of an Ig locus can be separately humanized and subsequently injected together into an animal recipient cell, wherein the overlapping fragments recombine in the recipient animal cell to generate a continuous Ig locus.

5   Human Ig gene segments can be integrated into the Ig locus on a vector (e.g., a BAC clone) by a variety of methods, including ligation of DNA fragments, or insertion of DNA fragments by homologous recombination. Integration of the human Ig gene segments is done in such a way that the human Ig gene segment is operably linked to the host animal sequence in the transgene to produce a functional humanized Ig locus, i.e., an 10   Ig locus capable of gene rearrangement and gene conversion which lead to the production of a diversified repertoire of humanized antibodies.

          Preferably, human Ig gene segments are integrated into the Ig locus by homologous recombination. Homologous recombination can be performed in bacteria, yeast and other cells with a high frequency of homologous recombination events. For 15   example, a yeast cell is transformed with a YAC containing an animal's Ig locus or a large portion thereof. Subsequently, such yeast cell is further transformed with a recombination vector as described hereinabove, which carries a human Ig gene segment linked to a 5' flanking sequence and a 3' flanking sequence. The 5' and the 3' flanking sequences in the recombination vector are homologous to those flanking sequences of the animal Ig gene 20   segment on the YAC. As a result of a homologous recombination, the animal Ig gene segment on the YAC is replaced with the human Ig gene segment. Alternatively, a bacterial cell such as *E. coli* is transformed with a BAC containing an animal's Ig locus or a large portion thereof. Such bacterial cell is further transformed with a recombination vector which carries a human Ig gene segment linked to a 5' flanking sequence and a 3' 25   flanking sequence. The 5' and the 3' flanking sequences in the recombination vector mediate homologous recombination and exchange between the human Ig gene segment on the recombination vector and the animal Ig gene segment on the BAC. Humanized YACs and BACs can be readily isolated from the cells and used in making transgenic animals.

          In a further aspect of the present invention, methods of making transgenic 30   animals capable of producing humanized immunoglobulins are provided.

According to the present invention, a transgenic animal capable of making humanized immunoglobulins are made by introducing into a recipient cell or cells of an animal one or more of the transgenic vectors described herein above which carry a humanized Ig locus, and deriving an animal from the genetically modified recipient cell or cells.

Preferably, the recipient cells are from non-human animals which generate antibody diversity by gene conversion and hypermutation, e.g., bird (such as chicken), rabbit, cows and the like. In such animals, the 3'proximal V gene segment is preferentially used for the production of immunoglobulins. Integration of a human V gene segment into the Ig locus on the transgene vector, either by replacing the 3'proximal V gene segment of the animal or by being placed in close proximity of the 3'proximal V gene segment, results in expression of human V region polypeptide sequences in the majority of immunoglobulins. Alternatively, a rearranged human V(D)J segment may be inserted into the J locus of the immunoglobulin locus on the transgene vector.

The transgenic vectors containing a humanized Ig locus is introduced into the recipient cell or cells and then integrated into the genome of the recipient cell or cells by random integration or by targeted integration.

For random integration, a transgenic vector containing a humanized Ig locus can be introduced into an animal recipient cell by standard transgenic technology. For example, a transgenic vector can be directly injected into the pronucleus of a fertilized oocyte. A transgenic vector can also be introduced by co-incubation of sperm with the transgenic vector before fertilization of the oocyte. Transgenic animals can be developed from fertilized oocytes. Another way to introduce a transgenic vector is by transfecting embryonic stem cells and subsequently injecting the genetically modified embryonic stem cells into developing embryos. Alternatively, a transgenic vector (naked or in combination with facilitating reagents) can be directly injected into a developing embryo. Ultimately, chimeric transgenic animals are produced from the embryos which contain the humanized Ig transgene integrated in the genome of at least some somatic cells of the transgenic animal.

In a preferred embodiment, a transgene containing a humanized Ig locus is randomly integrated into the genome of recipient cells (such as fertilized oocyte or developing embryos) derived from animal strains with an impaired expression of endogenous immunoglobulin genes. The use of such animal strains permits preferential

5   expression of immunoglobulin molecules from the humanized transgenic Ig locus. Examples for such animals include the Alicia and Basilea rabbit strains, as well as Agammaglobinemic chicken strain. Alternatively, transgenic animals with humanized immunoglobulin transgenes or loci can be mated with animal strains with impaired expression of endogenous immunoglobulins. Offspring homozygous for an impaired

10   endogenous Ig locus and a humanized transgenic Ig locus can be obtained.

For targeted integration, a transgenic vector can be introduced into appropriate animal recipient cells such as embryonic stem cells or already differentiated somatic cells. Afterwards, cells in which the transgene has integrated into the animal genome and has replaced the corresponding endogenous Ig locus by homologous recombination can be

15   selected by standard methods. The selected cells may then be fused with enucleated nuclear transfer unit cells, e.g. oocytes or embryonic stem cells, cells which are totipotent and capable of forming a functional neonate. Fusion is performed in accordance with conventional techniques which are well established. See, for example, Cibelli et al., Science (1998) 280:1256. Enucleation of oocytes and nuclear transfer can also be

20   performed by microsurgery using injection pipettes. (See, for example, Wakayama et al., Nature (1998) 394:369.) The resulting egg cells are then cultivated in an appropriate medium, and transferred into synchronized recipients for generating transgenic animals. Alternatively, the selected genetically modified cells can be injected into developing embryos which are subsequently developed into chimeric animals.

25   Further to the present invention, a transgenic animal capable of producing humanized immunoglobulins can also be made by introducing into a recipient cell or cells, one or more of the recombination vectors described herein above, which carry a human Ig gene segment, linked to 5' and 3' flanking sequences that are homologous to the flanking sequences of the endogenous Ig gene segment, selecting cells in which the endogenous Ig

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gene segment is replaced by the human Ig gene segment by homologous recombination, and deriving an animal from the selected genetically modified recipient cell or cells.

Similar to the target insertion of a transgenic vector, cells appropriate for use as recipient cells in this approach include embryonic stem cells or already differentiated

5   somatic cells. A recombination vector carrying a human Ig gene segment can be introduced into such recipient cells by any feasible means, e.g., transfection. Afterwards, cells in which the human Ig gene segment has replaced the corresponding endogenous Ig gene segment by homologous recombination, can be selected by standard methods. These genetically modified cells can serve as nuclei donor cells in a nuclear transfer procedure

10   for cloning a transgenic animal. Alternatively, the selected genetically modified embryonic stem cells can be injected into developing embryos which can be subsequently developed into chimeric animals.

Transgenic animals produced by any of the foregoing methods form another embodiment of the present invention. The transgenic animals have at least one, i.e., one

15   or more, humanized Ig loci in the genome, from which a functional repertoire of humanized antibodies is produced.

In a preferred embodiment, the present invention provides transgenic rabbits having one or more humanized Ig loci in the genome. The transgenic rabbits of the present invention are capable of rearranging and gene converting the humanized Ig loci,

20   and expressing a functional repertoire of humanized antibodies.

In another preferred embodiment, the present invention provides transgenic chickens having one or more humanized Ig loci in the genome. The transgenic chickens of the present invention are capable of rearranging and gene converting the humanized Ig loci, and expressing a functional repertoire of humanized antibodies.

25   Cells derived from the transgenic animals of the present invention, such as B cells or cell lines established from a transgenic animal immunized against an antigen, are also part of the present invention.

In a further aspect of the present invention, methods are provided for treating a disease in a primate, in particular, a human subject, by administering a purified humanized

antibody composition, preferably, a humanized polyclonal antibody composition, desirable for treating such disease.

The humanized polyclonal antibody compositions used for administration are generally characterized by containing a polyclonal antibody population, having

5   immunoglobulin concentrations from 0.1 to 100 mg/ml, more usually from 1 to 10 mg/ml. The antibody composition may contain immunoglobulins of various isotypes. Alternatively, the antibody composition may contain antibodies of only one isotype, or a number of selected isotypes.

In most instances the antibody composition consists of unmodified

10   immunoglobulins, i.e., humanized antibodies prepared from the animal without additional modification, e.g., by chemicals or enzymes. Alternatively, the immunoglobulin fraction may be subject to treatment such as enzymatic digestion (e.g. with pepsin, papain, plasmin, glycosidases, nucleases, etc.), heating, etc, and/or further fractionated.

The antibody compositions generally are administered into the vascular

15   system, conveniently intravenously by injection or infusion via a catheter implanted into an appropriate vein. The antibody composition is administered at an appropriate rate, generally ranging from about 10 minutes to about 24 hours, more commonly from about 30 minutes to about 6 hours, in accordance with the rate at which the liquid can be accepted by the patient. Administration of the effective dosage may occur in a single

20   infusion or in a series of infusions. Repeated infusions may be administered once a day, once a week once a month, or once every three months, depending on the half-life of the antibody preparation and the clinical indication. For applications on epithelial surfaces the antibody compositions are applied to the surface in need of treatment in an amount sufficient to provide the intended end result, and can be repeated as needed.

25   The antibody compositions can be used to bind and neutralize antigenic entities in human body tissues that cause disease or that elicit undesired or abnormal immune responses. An "antigenic entity" is herein defined to encompass any soluble or cell-surface bound molecules including proteins, as well as cells or infectious disease-causing organisms or agents that are at least capable of binding to an antibody and preferably are

30   also capable of stimulating an immune response.

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Administration of an antibody composition against an infectious agent as a
monotherapy or in combination with chemotherapy results in elimination of infectious
particles. A single administration of antibodies decreases the number of infectious
particles generally 10 to 100 fold, more commonly more than 1000-fold. Similarly,

5   antibody therapy in patients with a malignant disease employed as a monotherapy or in
combination with chemotherapy reduces the number of malignant cells generally 10 to
100 fold, or more than 1000-fold. Therapy may be repeated over an extended amount of
time to assure the complete elimination of infectious particles, malignant cells, etc. In
some instances, therapy with antibody preparations will be continued for extended periods

10   of time in the absence of detectable amounts of infectious particles or undesirable cells.
Similarly, the use of antibody therapy for the modulation of immune responses may
consist of single or multiple administrations of therapeutic antibodies. Therapy may be
continued for extended periods of time in the absence of any disease symptoms.

The subject treatment may be employed in conjunction with chemotherapy at

15   dosages sufficient to inhibit infectious disease or malignancies. In autoimmune disease
patients or transplant recipients, antibody therapy may be employed in conjunction with
immunosuppressive therapy at dosages sufficient to inhibit immune reactions.

The invention is further illustrated, but by no means limited, by the following
examples.

20

Example 1

Novel Sequences 3'prime of the Cγ Gene
from Cows, Sheep and Rabbits

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Genomic DNA was isolated from blood of a Simmental cow using the
QIAamp DNA Blood Kit (QIAGEN). The genomic region 3' of the cow Cγ gene (i.e., the
cow Cγ gene 3' flanking sequence) was PCR-amplified using the isolated genomic DNA

30   as template and the following primers:

5' primer: 5'cgcaagcttCCTACACGTGTGTGGTGATG3' (SEQ ID NO: 1);

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3' primer: 5'cgcaagcttAAGATGGWGATGGTSGTCCA3' (SEQ ID NO:

2)(Universal degenerate code: W=(A/T) S=(G/C)).

The upper-case portion of the 5' primer was from exon 3 of Cγ, and the lower-case

portion represented a terminal HindIII restriction site. The upper-case portion of the 3'

5   primer was a degenerate sequence designed according to the published sequences from the

human M1 exon and the mouse M1 exon, and the lower-case portion represented a

terminal HindIII restriction site. A 1.3kb PCR fragment was obtained using the EXPAND

long template PCR system (Roche). The fragment was gel purified, digested with

HindIII, and cloned into a Bluescript cloning vector. The resulting clones fell into three

10   populations, which differ from one another in the pattern of the restriction fragments

obtained with BamHI, EcoRI and XhoI. One clone from each population was sequenced,

and the sequences are shown in Figure 1 (SEQ ID NOS: 3-5).

Genomic DNA was isolated from blood of a Merino sheep using the QIAamp

DNA Blood Kit (QIAGEN). The genomic region 3' of the sheep Cγ gene (i.e., the sheep

15   Cγ gene 3' flanking sequence) was PCR-amplified using the isolated genomic DNA as

template and the following primers:

5' primer: 5'cgcggatccCCTACGCGTGTGTGGTGATG3' (SEQ ID NO: 6)

3' primer: 5'cgcggatccACCGAGGAGAAGATCCACTT3' (SEQ ID NO: 7)

The upper-case portion of the 5' primer was from exon 3 of Cγ, and the lower-case

20   portion represented a terminal BamHI restriction site. The upper-case portion of the 3'

primer was designed according to the published sequences from the human M2 exon and

the mouse M2 exon, and the lower-case portion represented a terminal BamHI restriction

site. A 2.9kb PCR fragment was obtained using the EXPAND long template PCR system

(Roche). The fragment was gel purified, digested with BamHI, and cloned into a

25   Bluescript cloning vector. The resulting clones fell into two populations, which differ

from each other in the pattern of the restriction fragments obtained with HindIII, EcoRI

and XhoI. One clone from each population was sequenced, and the sequences are shown

in Figure 2 (SEQ ID NOS: 8-9).

A 10kb EcoRI fragment containing the Cγ gene and its flanking sequences

30   from A2 allotype rabbit was subcloned from a genomic cosmid clone (cos 8.3 from

29

Knight et al., *J Immunol* (1985) 1245-50, "Organization and polymorphism of rabbit immunoglobulin heavy chain genes"). The nucleotide sequences 5' and 3' of Cγ were determined using standard methods and are set forth in Figure 3 and 5, SEQ ID NO: 10, 12, 13, respectively.

5   Sequences 3' of rabbit Ckappa1 were determined from an EcoRI/BamHI subclone from VJk2Ck In pSV2neo. The nucleotide sequence is set forth in Figure 4, SEQ ID NO: 11.

The amino acid sequences encoded by the M1 and M2 exons from cow, sheep and rabbit were deduced from the above 3' flanking sequence. These amino acid
10   sequences were aligned with the published M1 and M2 sequences from camel, human and mouse, as shown in Figure 6.

Example 2
A Vector for Replacing the Rabbit Endogenous Cγ
15   Gene Segment with the Human Cγ1 Segment

Genomic DNA is isolated from rabbit fetal fibroblasts of an a2-homozygous rabbit. The DNA sequence upstream of rabbit Cγ (i.e., the 5' flanking sequence of rabbit Cγ) is amplified by PCR using the following primers:
20   5' taattatgcggccgcCTTCAGCGTGAACCACGCCCTC 3' (SEQ ID NO: 39) with a 5' NotI site and
5' GTCGACGCCCCTCGATGCACTCCCAGAG 3' (SEQ ID NO: 40).

The DNA sequence downstream of rabbit Cγ (i.e., the 3' flanking sequence of rabbit Cγ) is amplified with the following primers:
25   5' ggtaccCTCTCCCTCCCCCACGCCGCAGC 3' (SEQ ID NO: 41) with a 5' KpnI site and
5' atatctcagaACTGGCTGTCCCTGCTGTAGTACACGG 3' (SEQ ID NO: 42) with a 5' XhoI site.

Human genomic DNA is isolated from human peripheral blood lymphocytes.
30   The DNA fragment encoding human Cγ1 is amplified using the following primers:
5' GTCGACACTGGACGCTGAACCTCGCGG 3' (SEQ ID NO: 43) and

5' GGTACCGGGGGCTTGCCGGCCGTCGCAC 3' (SEQ ID NO: 44).

The fragments are digested with restriction enzymes and cloned into a

Bluescript vector. Subsequently, a lox neo-cassette is inserted into the SalI site and an

Hsv-tk cassette into the XhoI site. A schematic drawing of the final construct is shown in

5   Figure 7a.

Example 3
A Vector for Replacing the Rabbit Endogenous Cκ
Gene Segment with the Human Cκ Segment

10

Genomic DNA was isolated from rabbit fetal fibroblasts of a b5-homozygous

rabbit. The DNA sequence upstream of rabbit Cκ1 (i.e., the 5' flanking sequence of rabbit

Cκ1) was amplified by PCR using the following primers:

5' gcggccgcTGGCGAGGAGACCAAGCTGGAGATCAAACG 3' (SEQ ID

15   NO: 45) with a 5' NotI site

5' GTCGACGCAGCCCAAAGCTGTTGCAATGGGGCAGCG 3' (SEQ ID

NO: 46).

The DNA sequence downstream of rabbit Cκ1 (i.e., the 5' flanking sequence

of rabbit Cκ1) was amplified with the following primers:

20   5' atatggtaccGCGAGACGCCTGCCAGGGCACCGCC 3' (SEQ ID NO: 47)

with a 5' KpnI site

5' GGATCCCGAGCTTTATGGGCAGGGTGGGGG 3' (SEQ ID NO: 48).

Human genomic DNA was isolated from human peripheral blood

lymphocytes. The DNA fragment encoding human Cκ was amplified using the following

25   primers:

5' ATATGTCGACCTGGGATAAGCATGCTGTTTTCTGTCTGTCCC 3'

(SEQ ID NO: 49)

5' CTAGGTACCAGCAGGTGGGGGCACTTCTCCC 3' (SEQ ID NO: 50).

The fragments were digested with restriction enzymes and cloned into a Bluescript vector.

30   Subsequently, a lox neo-cassette was inserted into the SalI site and an Hsv-tk cassette into

the XhoI site. A schematic drawing of the final construct is shown in Figure 7b.

31

Example 4

Replacement of the Endogenous Cγ and Cκ Gene Segments in Rabbit Fetal Fibroblasts with the Corresponding Human Gene Segments

Rabbit fetal fibroblast cells are prepared by standard methods. After one passage, fibroblasts are transfected with 5μg of the NotI-linearized targeting vector as shown in Figure 5a for Cγ or Figure 51b for Cκ, and are seeded in 96-well plates (2 x 10^3 cells/well). After a positive selection with 600μg/ml G418 and a negative selection with 200nM FIAU, resistant colonies are replica-plated to two 96-well plates for DNA analysis and cryopreservation, respectively. PCR and/or Southern blot analysis is performed to identify cells with the human Cγ1 gene segment integrated in the genome. The cells having the integrated human Cγ1 gene are used in rabbit cloning as described in Example 5.

Example 5

Cloning of Rabbits

Mature Dutch Belton rabbits are superovulated by subcutaneous injection of follicle stimulating hormone (FSH) every 12 hours (0.3 mg x 2 and 0.4 mg x 4). Ovulation is induced by intravenous administration of 0.5 mg luteinizing hormone (LH) 12 hours after the last FSH injection. Oocytes are recovered by ovidual flush 17 hours after LH injection. Oocytes are mechanically enucleated 16-19 hours after maturation. Chromosome removal is assessed with bisBENZIMIDE (HOECHST 33342, Sigma, St. Louis, MO) dye under ultraviolet light. Enucleated oocytes are fused with actively dividing fibroblasts by using one electrical pulse of 180 V/cm for 15 us (Electrocell Manipulator 200, Genetronics, San Diego, CA). After 3-5 hours oocytes are chemically activated with calcium ionophore (6 uM) for 4 min (# 407952, Calbiochem, San Diego, CA) and 2 mM 6-dimethylaminopurine (DMAP, Sigma) in CR2 medium (Specialty Media, Lavalett, NJ) with 3 mg/ml bovine serum albumin (fatty acid free, Sigma) for 3 hours. Following the activation, the embryos are washed in hamster embryo culture

medium (HECM)-Hepes five times and subsequently, cultivated in CR2 medium containing 3 mg/ml fatty-acid free BSA for 2-48 hours at 37.8° C and 5%CO_2 in air. Embryos are then transferred into synchronized recipients. Offsprings are analyzed by PCR for a segment of the transgene.

5

Example 6
Construction of a DNA Fragment Containing a Portion of a Rabbit Heavy Chain Locus with a Human Cγ1 Gene Segment and a VH Gene
10   Segment Encoding a Human VH Domain Polypeptide Sequence

The upstream and downstream regions (i.e., the 5' and 3' flanking regions) of the rabbit heavy chain Cγ gene from an a2-allotype rabbit were sequenced. A DNA fragment (SEQ ID NO: 51) is generated by PCR using overlapping oligonucleotides

15   wherein the DNA fragment contains from 5' to 3', a sequence derived from the 5' flanking region of the rabbit Cγ gene, the human Cγ1 gene, and a sequence derived from the 3' flanking region of the rabbit Cγ gene (Figure 8).

A genomic BAC library derived from an a2-allotype rabbit is generated by standard procedures and screened with probes specific for rabbit Cγ. A BAC clone

20   containing rabbit heavy chain gene segments is identified. The rabbit Cγ gene on this BAC clone is replaced with the human Cγ1 gene by homologous recombination in *E.coli* using the DNA fragment of SEQ ID NO: 51 and the pET system. This replacement is accomplished by two consecutive recombination steps: first the rabbit Cγ gene segment is replaced with a marker gene; then the marker gene is replaced the human Cγ1 gene

25   segment.

The modified BAC clone containing rabbit heavy chain genes and the inserted human Cγ1 gene is further modified by replacing the 3'proximal VH1 segment with a synthetic VH gene segment (Figure 9). This synthetic VH gene segment (SEQ ID NO: 52) is made using overlapping oligonculeotides and includes a 5' flanking sequence, a 3'

30   flanking sequence, and a sequence coding for a polypeptide nearly identical to the human immunoglobulin heavy chain variable domain polypeptide sequence described by Huang

and Stollar (*J. Immunol.* 151: 5290-5300, 1993). The coding sequence of the synthetic VH gene segment is designed based on the published sequence of a rabbit VH1 gene (a2, Knight and Becker, *Cell* 60:963-970, 1990) and is more than 80% identical to rabbit VH gene segments. The 5' and the 3' flanking sequences in the synthetic VH segment are

5   derived from the upstream and downstream regions of the a2-allotype rabbit VH1 gene. The synthetic VH gene of SEQ ID NO: 52 is used to replace the rabbit VH1 gene on the BAC clone by homologous recombination using the pET or the redεβγ system. The modified BAC clone is amplified and purified using standard procedures.

10

Example 7

Construction of a DNA Fragment Containing a Portion of a Rabbit Light Chain Locus with a Human Cκ Gene Segment and a VJ Gene Segment Encoding a Human VL Domain Polypeptide Sequence

15
The upstream and downstream regions (i.e., the 5' and 3' flanking regions) of the rabbit light chain Cκ1 gene from a b5-allotype rabbit were sequenced. A DNA fragment (SEQ ID NO: 53) is generated by PCR using overlapping oligonucleotides wherein the DNA fragment contains from 5' to 3', a sequence derived from the 5'

20   flanking region of the rabbit Cκ1 gene, the human Cκ1 gene, and a sequence derived from the 3' flanking region of the rabbit Cκ1 gene (Figure 10).

A genomic BAC library derived from a b5-allotype rabbit is generated by standard procedures and screened with probes specific for rabbit Cκ1. A BAC clone containing rabbit light chain gene segments is identified. The rabbit Cκ1 gene on this

25   BAC clone is replaced with the human Cκ1 gene on the DNA fragment of SEQ ID NO: 53 by homologous recombination in *E.coli* using the pET or the redεβγ system. This replacement is accomplished by two consecutive recombination steps: first the rabbit Cκ1 gene segment is replaced with a marker gene; then the marker gene is replaced the human Cκ1 gene segment.

30   The modified BAC clone containing rabbit light chain genes and the inserted human Cκ1 gene is further modified by inserting a rearranged VJ DNA fragment into the

J region of the rabbit light chain locus. The rearranged VJ DNA fragment encodes a
human immunoglobulin variable domain polypeptide described by Pritsch et al. (*Blood*
82(10):3103-3112, 1993) and Lautner-Rieske et al. (*Eur. J. Immunol.* 22 (4), 1023-1029,
1992)) (Figure 7). The nucleotide sequence of the rearranged VJ fragment is designed to

5   maximize the sequence homology at the nucleotide level to the rabbit Vkappa sequence
published by Lieberman et al. (*J. Immunol.* 133 (5), 2753-2756, 1984). This rearranged
VJ DNA sequence is more than 80% identical with known rabbit Vκ genes. Using
overlapping oligonucleotides in PCR, the rearranged VJ DNA fragment is linked to a 5'
and a 3' flanking sequence, resulting the DNA fragment of SEQ ID NO: 54 (Figure 11).

10   The 5'flanking sequence is derived from 5' of a rabbit Vκ, the 3'flanking sequence is
derived from 3' of rabbit J2. The DNA fragment of SEQ ID NO: 54 is subsequently
inserted into the rabbit light chain locus by homologous recombination in *E. coli* using the
pET or the redεβγ system. The insertion is performed in such a way that the rabbit light
chain region containing the rabbit Vκ1 gene segment, the rabbit J1 and J2 segments, and

15   the sequences in between, is replaced with the rearranged VJ DNA fragment. Again, this
insertion is accomplished by replacement of the rabbit V to J region with a marker gene,
followed by the replacement of the marker gene with the rearranged VJ DNA fragment.
The modified BAC clone is amplified and purified using standard procedures.

20

Example 8
Transgenic Rabbits Expressing the Humanized
Immunoglobulin Light and/or Heavy Chain Transgene

25   Transgenic rabbits are generated as described by Fan et al. (*Pathol. Int.* 49:
583-594, 1999). Briefly, female rabbits are superovulated using standard methods and
mated with male rabbits. Pronuclear-stage zygotes are collected from oviduct and placed
in an appropriate medium such as Dulbecco's phosphate buffered saline supplemented
with 20% fetal bovine serum. The exogenous DNA (e.g., the humanized BAC clone from

30   Example 4 and/or 5 which has been linearized prior to injection) is microinjected into the
male pronucleus with the aid of a pair of manipulators. Morphological surviving zygotes

are transferred to the oviducts of pseudopregnant rabbits. Pseudopregnancy is induced by the injection of human chorionic gonadotrophin (hCG). Between about 0.1-1% of the injected zygotes develop into live transgenic rabbits. Integration of the transgene in the genome is confirmed by Southern blots analysis using a probe specific for the transgene.

5   cDNA is prepared using RNA isolated from B cells (in blood, spleen and/or lymph nodes) of a transgenic rabbit. Primers specific for the human transgene (human CH gene segment or the synthetic humanized VH gene segment) are used to generate amplified products from cDNA. The observation of amplified products indicates that the transgene is rearranged in the transgenic animal and the rearranged transgene is

10   transcribed in the animal. Amplified products are sequenced and the presence of donor sequences from upstream V genes indicates that the transgene introduced into the germline of the animal undergoes gene conversion.

The presence of antibodies containing human IgG and/or human kappa light chain antigenic determinants in the serum of transgenic founder rabbits is determined

15   using an ELISA assay.

20

Example 9

Production of Humanized Antibodies From Transgenic Rabbits
with the Genetic Background of the Alicia and/or Basilea Rabbit Strain

25   The Alicia strain lacks the VH1 gene segment and therefore has an impaired Ig heavy chain expression. Transgenic founder rabbits capable of expressing humanized heavy chain molecules in the genetic background of the Alicia rabbit strain are generated, e.g., by using fetal fibroblasts established from Alicia rabbits in Examples 4-5 above, or by using zygotes from female Alicia rabbits mated with male Alicia rabbits in Example 8

30   above. Transgenic animals are also obtained which are homozygous for the Alicia Ig phenotype and are also homozygous for a humanized heavy chain transgene. Serum is tested in ELISA for the presence of humanized heavy chain (e.g., a human heavy chain

constant region). The concentration of antibodies with humanized Ig heavy chains in these homozygous Alicia animals is substantially higher, e.g., about 10 to 100 fold higher, than that produced from a transgene integrated in the genome of wild type (non-Alicia) rabbits.

5   The Basilea strain does not express κ1 light chain and in its place exclusively express the κ2 and λ light chains. Transgenic founder rabbits capable of expressing humanized light chain molecules in the genetic background of the Basilea rabbit strain are generated, e.g., by using fetal fibroblasts established from Basilea rabbits in Examples 4-5 above, or by using zygotes from female Basilea rabbits mated with male Basilea rabbits in

10   Example 8 above. Transgenic animals are obtained which are homozygous for the Basilea light chain phenotype, and are also homozygous for a humanized light chain transgene. Serum is tested in ELISA for the presence of the humanized light chain. The concentration of the humanized light chain in the homozygous Basilea animals is substantially higher, about 10-100 fold higher, than the concentration of a humanized light

15   chain in a transgenic rabbit with the wild type (non-Basilea) genetic background. Transgenic founder rabbits are mated with each other to generate transgenic rabbits with the following traits: (1) having at least one humanized light chain transgene, (2) having at least one humanized heavy chain transgene, (3) homozygous for the Alicia heavy chain locus, and (4) homozygous for the Basilea light chain locus.

20

Example 10
Construction of a DNA Fragment Containing a Modified Chicken Light Chain Locus Having a Human Clambda2 Gene Segment and a VJ Gene Segment Encoding a Human VL Domain

25

A genomic BAC library derived from a jungle fowl chicken was screened with radiolabeled probes specific for chicken light chain Clambda and chicken Vpsi25 (the V gene segment at the very 5' end of the light chain locus). A BAC clone containing the entire lambda light chain locus was identified. The chicken Cλ gene on this BAC clone is

30   replaced with the human Cλ2 gene by homologous recombination in *E.coli* using the pET system (Zhang et al., *Nat. Biotechnol.* 18(12):1314-7, 2000) as follows.

37

A first DNA fragment containing a kanamycin selection cassette was generated
by PCR using primers specific for Tn5 gene. The 5' primer
(5'catacacagccatacatacgcgtgtggccgctctgcctctctcttgcaggTATGGACAGCAAGCGAACCG
3', SEQ ID NO: 55) was designed to include 50 bp at the 5' end (lower case), derived

5   from the 5' flanking region of the chicken light chain Cλ gene. The 3' primer
(5'atcagggtgaccccctacgttacactcctgtcaccaaggagtgggagggacTCAGAAGAACTCGTCAAGA
AG3', SEQ ID NO: 56) was designed to include about 50 bp at the end (lower case),
derived from the 3' flanking region of the chicken light chain Cλ gene.

A second DNA fragment (SEQ ID NO: 57) was synthesized using overlapping

10 oligonucleotides wherein the DNA fragment contains from 5' to 3', a sequence derived
from the 5' flanking region of the chicken light chain Clambda gene, the human
Clambda2 gene, and a sequence derived from the 3' flanking region of the chicken
Clambda gene (Figure 12).

E. coli cells of the chicken light chain BAC clone were transformed with a

15 recombination plasmid expressing the recE and recT functions under an inducible
promotor. Cells transformed with the recombination plasmid were then transformed with
the first DNA fragment above and selected afterwards in media containing kanamycin.
Clones resistant to kanamycin were identified, and the replacement of the chicken Cλ
segment by the kanamycin selection cassette via homologous recombination was

20 confirmed by restriction enzyme digest.

In the second homologous recombination step, cells positive for the kanamycin
selection cassette were transformed with the second DNA fragment above. Transformed
cells were screened for the loss of kanamycin resistance as indicative of the replacement
of the kanamycin selection cassette by the human Cλ2 gene. The exchange was

25 confirmed by restriction enzyme digest and/or sequence analysis.

The ET cloning procedure is summarized in Figure 13.

The BAC clone containing the chicken light chain locus and the inserted
human Clambda2 gene segment was further modified by inserting a rearranged VJ DNA
fragment. The rearranged VJ DNA fragment encodes a human immunoglobulin variable

30 domain polypeptide described by Kametani et al. (*J. Biochem.* 93 (2), 421-429, 1983) as

IG LAMBDA CHAIN V-I REGION NIG-64 (P01702) (Figure 14). The nucleotide
sequence of the rearranged VJ fragment was so designed as to maximize the sequence
homology at the nucleotide level to the chicken Vlambda1 sequence published by
McCormack et al. (*Cell* 56, 785-791, 1989). This rearranged VJ DNA sequence is more

5   than 80% identical with known chicken light chain V genes. The rearranged VJ DNA
fragment was linked to a 5' flanking sequence and a 3' flanking sequence, resulting in the
DNA fragment of SEQ ID NO: 58 (Figure 14). The 5' flanking sequence was derived
from 5' of chicken Vlambda1, and the 3'flanking sequence was derived from 3' of
chicken J. The DNA fragment of SEQ ID NO: 58 was subsequently inserted into the

10   chicken light chain locus in *E. coli* using the pET system as shown in Figure 15. The
insertion was performed in such a way that the region on the chicken light chain locus
from the 5' end of the chicken Vlambda1 gene segment to the 3' end of the chicken J
region was replaced with the rearranged, synthetic VJ DNA fragment. Again, this
insertion wais accomplished by the replacement of the chicken V-J region with a marker

15   gene, followed by the replacement of the marker gene with the rearranged VJ DNA
fragment. The modified region of the chicken light chain locus is shown in Figure 15.
The modified BAC clone was amplified and purified using standard procedures.

Example 11

20   **Construction of a DNA Fragment Containing a Portion of a Chicken
Heavy Chain Locus With a Human Cγ1 Gene Segment and a VH Gene
Segment Encoding a Human VH Domain Polypeptide Sequence**

A jungle fowl chicken genomic BAC library was generated by standard
procedures and screened with probes specific for chicken Cγ. A BAC clone containing

25   chicken heavy chain gene segments is identified. The upstream and downstream regions
(i.e., the 5' and 3' flanking regions) of the heavy chain Cγ gene are sequenced. The
chicken Cγ gene on this BAC clone is replaced with the human Cγ1 gene by homologous
recombination in *E.coli* using the pET system as follows.

A first DNA fragment containing a kanamycin selection cassette is generated

30   by PCR using primers specific for Tn5 gene. The 5' and 3' primers are designed to

39

include about 50 bp at the end, derived from the 5' and 3' flanking regions of the chicken heavy chain Cγ gene.

A second DNA fragment is generated by PCR using overlapping oligonucleotides wherein this second DNA fragment contains from 5' to 3', a sequence of

5   about 50 bp derived from the 5' flanking region of the chicken Cγ gene, the human Cγ1 gene, and a sequence of about 50 bp derived from the 3' flanking region of the chicken Cγ gene.

E. coli cells of the chicken CY BAC clone are transformed with a recombination plasmid expressing the recE and recT functions under an inducible

10   promotor. Cells transformed with the recombination plasmid are further transformed with the first DNA fragment and selected in media containing kanamycin. Clones resistant to kanamycin are identified, and the replacement of the chicken CY segment by the kanamycin selection cassette via homologous recombination is confirmed by restriction enyme digest.

15   In the second homologous recombination step, cells positive for the kanamycin selection cassette are now transformed with the second DNA fragment described above. Transformed cells are screened for loss of kanamycin resistance as indicative of the replacement of the kanamycin selection cassette by the human Cγ1 gene. The exchange is confirmed by restriction enzyme digest and/or sequence analysis.

20   The BAC clone containing the inserted human Cγ1 gene is further modified by replacing the 3'proximal VH1 segment (i.e., the 3'proximal VH1 gene in the V region) with a synthetic VH gene segment. This synthetic VH gene segment is designed based on the published sequence of a chicken VH1 gene (Arakawa et al., EMBO J 15(10): 2540-2546, 1996). The synthetic gene segment is more than 80% identical to chicken VH gene

25   segments and encodes an amino acid sequence that is identical to the amino acid sequence of a human immunoglobulin heavy chain variable domain polypeptide described by Matthyssens and Rabbitss (in Steinberg CM and Lefkovits I, (eds). *The Immune System*: 132-138, S. Karger, NY 1981). This synthetic VH segment including 5' and 3' flanking sequences is synthesized by PCR using overlapping oligonucleotides. The 5' and the 3'

30   flanking sequences are derived from the upstream and downstream regions of chicken

VH1 gene. This synthetic VH segment is used to replace the chicken VH1 gene on the
BAC clone by homologous recombination using the pET system. The modified BAC
clone is amplified and purified using standard procedures.

5

Example 12

Transgenic Chicken Expressing the Humanized
Immunoglobulin Light and/or Heavy Chain Transgenes

The production of transgenic chicken is carried out using techniques as
10   described by Etches et al., *Methods in Molecular Biology* 62: 433-450; Pain et al., *Cells
Tissues Organs* 1999; 165(3-4): 212-9; Sang, H., "Transgenic chickens--methods and
potential applications", *Trends Biotechnol 12:415* (1994); and in WO 200075300,
"Introducing a nucleic acid into an avian genome, useful for transfecting avian
blastodermal cells for producing transgenic avian animals with the desired genes, by
15   directly introducing the nucleic acid into the germinal disc of the egg".

Briefly, the modified BAC clones are linearized and mixed with a transfection
reagent to promote uptake of DNA into cells. The formulations are injected into a
multicell stage chicken embryo in close proximity to the germinal disc. The window in
the egg shell is closed and the eggs are incubated. After hatching chimeric chickens are
20   identified by PCR and Southern blot analysis using transgene specific sequences.
Integration of the transgene in the genome is confirmed by Southern blots analysis using a
probe specific for the transgene. Heavy and light chain transgenic animals are bred with
each other to generate transgenic chickens expressing antibodies having humanized heavy
and light chains.

25   cDNA is prepared using RNA isolated from B cells (in blood, spleen and/or
lymph nodes) from transgenic chickens. Primers specific for the human transgene (e.g.,
human CH gene segments and/or the synthetic humanized VH gene segments) are used to
generate amplified products from cDNA. The observation of amplified products indicates
that the transgene is rearranged in the transgenic animal and the rearranged transgene is
30   transcribed in the animal. Amplified products are sequenced and the presence of donor

41

sequences from upstream V genes indicates that the transgene introduced into the
germline of the animal undergoes gene conversion.

The presence of antibodies containing human IgG and/or human kappa light
chain antigenic determinants in the serum of transgenic chickens is determined using an
5   ELISA assay.

Example 13

Production of Functional Humanized Antibodies
in Transgenic Chicken with the Agammaglobulinemic Phenotype
10

Transgenic chickens with the following traits are produced: (1) having at least
one humanized light chain transgene, (2) having at least one humanized heavy chain
transgene, and (3) homozygous for the agammaglobulinemic phenotype. These animals
produce antibodies into the blood and eggs, and antibodies can be purified from either
15   source. In general, antibody concentrations in the eggs are about 5% to 50% of antibodies
concentration in the blood. Animals that contain humanized antibodies at high levels in
eggs can be selected and bred to produce offspring. Alternatively, transgenic animals can
be generated that specifically secrete humanized antibodies into their eggs.

20

Example 14

Generation Of Transgenic Chickens
Expressing Humanized Immunoglobulin

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Chicken embryonic stem cells are isolated and cultured as described by Pain et
al. (*Development* 122, 2339-2348; 1996). Chicken embryos are obtained from eggs
immediately after they are laid. The entire blastoderm is removed by gentle aspiration,
embryos are slowly dissociated mechanically and cells are seeded in ESA complete
30   medium on inactivated STO feeder cells. ESA medium is composed of MEM medium
containing 10% FCS, 2% chicken serum, 1% bovine serum albumin, 10 ng/ml ovalbumin,
1 mM sodium pyruvate, 1% non-essential amino acids, 1 μM of each nucleotide

adenosine, guanosine, cytidine, uridine, thymidine, 0.16 mM β-mercaptoethanol, ESA complete medium is supplemented with 10 ng/ml bFGF, 20 ng/ml h-IGF-1, 1% vol/vol avian-SCF and 1% vol/vol h-LIF, 1% vol/vol h-IL-11. Cell cultures are incubated wt $37°C$ in 7.5 CO_2 and 90% humidity. After 48 hours fresh blastodermal cells are added to

5   the culture in half of the original volume of ESA complete medium. After an additional incubation for three days, the culture medium is partially (50%) replaced with fresh ESA complete medium, and totally every day thereafter. For cell harvesting, cultures are washed with PBS and incubated in a pronase solution (0.025% w/v). Dissociated cells are transfected with various linearized transgenic constructs containing a humanized Ig locus.

10   Transfected cells are incubated with STO feeder cells (as described above) in the presence of selective antibiotics. Cells are transferred onto fresh feeder cells twice per week. Antibiotic resistant cells are isolated and the integration of a humanized Ig gene fragments at a random site or at the corresponding chicken immunoglobulin gene loci is confirmed by PCR.

15   Subsequently, genetically modified cells are injected into recipient embryos. As recipient embryos, freshly laid eggs are irradiated (6Gy - Cobalt source). Between 100 to 200 genetically modified cells are injected into the subgerminal cavity using a micropipet. The window in the egg shell is closed and the eggs are incubated. Somatic chimerism of hatched chickens is evaluated by PCR. Germ-line chimerism is assessed by

20   mating of somatic chimeras.

Example 15
Immunization Of Transgenic Animals

25   Genetically engineered chickens are immunized intramuscularly with purified Hepatitis B surface antigen (HBsAg) (5μg in incomplete Freund's adjuvant) on day 0, 14 and day 28. On day 35 animals are bled and serum is prepared. ELISA plates (NUNC, Denmark) are coated with 1 μg/ml HBsAg in PBS for 1 hour at room temperature. Subsequently, available binding sites are blocked by incubation with 1% non-fat dry milk

30   (NFM) in PBS (300 μl/well). Chicken serum is diluted in PBS/1%NFM and added to the

coated wells. After an incubation of 1 hour, the plates are washed 3 times with
PBS/0.05% Tween 20 and bound Ig is detected using goat anti-human Ig conjugated with
horseradish peroxidase. Conjugated goat antibody is detected using o-phenylenediamine
dihydrochloride (Sigma) at 1 mg/ml. The colorimetric reaction is stopped by addition of 1

5   M HCl solution and the absorbance is measured at 490 nm. As a control, serum from
non-immunized chicken is used. Serum from non-immunized chickens does not react
with HBsAg. At a dilution of 1:250 the optical density measured in uncoated and HBsAg
coated wells is below 0.2. In contrast, serum from immunized chickens contains
humanized antibodies reactive with HBsAg. At a serum dilution of 1:250 the measured

10   optical density is 2.3. Upon further dilution of the serum the measured optical density
declines to 0.1 (at a dilution of 25600). No antibodies reactive with a goat anti-chicken
IgG-HRP conjugate can be detected. This demonstrates that the genetically engineered
chickens produce humanized anti-HBsAg antibodies following immunization.

        Genetically engineered rabbits are immunized intramuscularly with purified
15   Hepatitis B surface antigen (HBsAg) (10μg in incomplete Freund's adjuvant) on day 0 and
day 14. On day 28 animals are bled from the ear and serum is prepared. ELISA plates
(NUNC, Denmark) are coated with 1 μg/ml HBsAg in PBS for 1 hour at room
temperature. Subsequently, available binding sites are blocked by incubation with 1%
non-fat dry milk (NFM) in PBS (300 μl/well). Rabbit serum is diluted in PBS/1%NFM

20   and added to the coated wells. After an incubation of 1 hour, the plates are washed 3 times
with PBS/0.05% Tween 20 and bound Ig is detected using goat anti-human Ig conjugated
with horse-radish peroxidase. Conjugated goat antibody is detected using
o-phenylenediamine dihydrochloride (Sigma) at 1 mg/ml. The colorimetric reaction is
stopped by addition of 1 M HCl solution and the absorbance is measured at 490 nm. As a

25   control serum from non-immunized rabbits is used. Serum from non-immunized rabbits
does not react with HBsAg. At a dilution of 1:100 the optical density measured in
uncoated and HBsAg coated wells is below 0.4. In contrast, serum from immunized
rabbits contains partially human antibodies reactive with HBsAg. At a serum dilution of
1:100 the measured optical density is 2.8. Upon further dilution of the serum the

30   measured optical density declines to 0.2 (at a dilution of 25600). No antibodies reactive

                                          44

with a goat anti-rabbit IgG-HRP conjugate can be detected. This demonstrates that the genetically engineered rabbits produce humanized anti-HBsAg antibodies following immunization.

5

Example 16

Complement Mediated Cytotoxicity of Virus Infected Cell Line Using Humanized Antibodies

A human liver carcinoma cell line expressing HBsAg is labeled with 0.1 mCi 10 ^{51}Cr in 100 ul PBS for 1 hr at 37°C. Two thousand ^{51}Cr-lableled cells are incubated with serum from genetically engineered rabbits or chickens expressing anti-HbsAg humanized immunoglobulins. After two hours at 37°C the release of ^{51}Cr into the supernatant is determined by measuring radioactivity using a scintillation counter. For the determination of maximum release, 1% Triton X100 is added. The degree of cell lysis is calculated as 15 follows: %Lysis = CPM experimental ±CPM#spontaneous / CPM# total ± CPM spontaneous. Incubation of labeled cells with serum (diluted 1:30) from non-immunized rabbits does not result in cell lysis (<10%). However, incubation of cells with serum from immunized rabbits causes 80% cell lysis. Inactivation of complement in the serum by heat treatment (56°C for 30 minutes) renders the serum from immunized rabbits inactive. 20 These results demonstrate that humanized antibodies produced by genetically engineered rabbits bind to HBsAg-positive cells and cause complement dependent lysis.

Example 17

Immunization of Transgenic Animals against Staphylococcus aureus

25

Genetically engineered chickens are immunized intramuscularly with a recombinant fragment of the Staphylococcus aureus collagen adhesin protein (100μg in incomplete Freund's adjuvant) on day 0, 14 and day 28. On day 35 animals are bled and serum is prepared. ELISA plates (NUNC, Denmark) are coated with 2 μg/ml collagen 30 adhesin protein in PBS for 1 hour at room temperature. Subsequently, available binding sites are blocked by incubation with 1% non-fat dry milk (NFM) in PBS (300 μl/well).

Chicken serum is diluted in PBS/1%NFM and added to the coated wells. After an

incubation of 1 hour, the plates are washed 3 times with PBS/0.05% Tween 20 and bound

Ig is detected using goat anti-human Ig conjugated with horseradish peroxidase.

Conjugated goat antibody is detected using o-phenylenediamine dihydrochloride (Sigma)

5   at 1 mg/ml. The colorimetric reaction is stopped by addition of 1 M HCl solution and the

absorbance is measured at 490 nm. As a control, serum from non-immunized chicken is

used. Serum from non-immunized chickens does not react with collagen adhesin protein.

At a dilution of 1:250 the optical density measured in uncoated and collagen adhesin

protein coated wells is below 0.2. In contrast, serum from immunized chickens contains

10   humanized antibodies reactive with collagen adhesin. At a serum dilution of 1:250 the

measured optical density is 2.3. Upon further dilution of the serum the measured optical

density declines to 0.1 (at a dilution of 25600). No antibodies reactive with a goat

anti-chicken IgG-HRP conjugate can be detected. This demonstrates that the genetically

engineered chickens produce humanized anti-Staph. aureus collagen adhesin antibodies

15   following immunization.

Genetically engineered rabbits are immunized intramuscularly with

recombinant fragment of the Staphylococcus aureus collagen adhesin protein (100µg in

incomplete Freund's adjuvant) on day 0 and day 14. On day 35 animals are bled and

serum is prepared. ELISA plates (NUNC, Denmark) are coated with 2 µg/ml collagen

20   adhesin protein in PBS for 1 hour at room temperature. Subsequently, available binding

sites are blocked by incubation with 1% non-fat dry milk (NFM) in PBS (300 µl/well).

Rabbit serum is diluted in PBS/1%NFM and added to the coated wells. After an

incubation of 1 hour, the plates are washed 3 times with PBS/0.05% Tween 20 and bound

Ig is detected using goat anti-human Ig conjugated with horseradish peroxidase.

25   Conjugated goat antibody is detected using o-phenylenediamine dihydrochloride (Sigma)

at 1 mg/ml. The colorimetric reaction is stopped by addition of 1 M HCl solution and the

absorbance is measured at 490 nm. As a control, serum from non-immunized rabbit is

used. Serum from non-immunized rabbits does not react with collagen adhesin protein.

At a dilution of 1:250 the optical density measured in uncoated and collagen adhesin

30   protein coated wells is below 0.2. In contrast, serum from immunized rabbits contains

46

humanized antibodies reactive with collagen adhesin. At a serum dilution of 1:250 the measured optical density is 2.3. Upon further dilution of the serum the measured optical density declines to 0.1 (at a dilution of 25600). No antibodies reactive with a goat anti-rabbit IgG-HRP conjugate can be detected. This demonstrates that the genetically

5   engineered rabbits produce humanized anti-Staph. aureus collagen adhesin antibodies following immunization.

Example 18
Protection Against *Staphylococcus Aureus* Infection In A Mouse Model

10

Naive mice are passively immunized i.p. on day -1 with 16 mg of the immunoglobulin fraction containing antibodies specific for the *S. aureus* collagen adhesin protein (from Example 17) or with the immunoglobulin fraction from non-immunized animals. On day 0, the mice are challenged i.v. with 4×10^7 CFU *S. aureus* per mouse and

15   mortality is monitored over the next 7 days. Mortality rate in the control groups is 80% and 10% in the group treated with the immunoglobulin fraction containing antibodies specific for the *S. aureus* collagen adhesin protein. The data indicate that anticollagen adhesin antibodies can protect mice against lethal *S. aureus* challenge.

20   ## Example 19
Antigen-Specific Hybridomas Made From Transgenic Animals.

Transgenic animals are immunized with an antigen (e.g., KLH, human red blood cells or sheep red blood cells). Spleen cells are removed at various times after

25   immunization and fused with myeloma cell lines derived from rabbit and chicken, respectively. After fusion cells are plated into 96 well plates and supernatants are tested for the presence of humanized antibodies. To demonstrate that the antibodies contain human immunoglobulin sequences, hybridomas are stained with fluorescent-labeled antibodies reactive with human heavy and light chain immunoglobulins. Limiting dilution

30   is conducted to purify hybridomas to monoclonality.

47

Example 20
Evaluation of Immunogenicity

5   Serum samples are collected from five cynomologous monkeys on day 0.
Subsequently, a purified partially human polyclonal antibody preparation (5 mg/kg) is
administered into five cynomologous monkeys by intravenous administration. The
administration is repeated six times in bi-weekly intervals. Monkeys are monitored
closely for any side-effects (e.g., anaphylactic shock, reflected by an elevated body
10   temperature). After seven months serum is collected from blood samples. Affinity resins
containing purified human IgG or partially human IgG are produced by standard
procedure using CNBr-activated Sepharose. Monkey serum samples (3 ml) are added to
the IgG-affinity resin (4 ml) containing 10 mg human or partially human IgG.
Subsequently, the columns are washed with PBS. Bound monkey immunoglobulin is
15   eluted from the column with 0.1M glcyin/HCl pH2.5 and dialyzed 2 times against PBS.
The protein content of the eluted fractions is determined using the BCA assay using
human IgG as a standard. The total amounts of protein in these fractions demonstrate that
therapy with partially human IgG does not lead to a significant antibody response in the
treated animals.

20   ## Example 21
Treating Animals Using Humanized Antibodies

        Humanized polyclonal immunoglobulins are purified from the serum of
genetically engineered rabbits, or from egg yolk of genetically engineered chickens, by
25   ammonium sulfate precipitation and ion exchange chromatography. SCID-mice are
injected with one million human liver carcinoma cells expressing HBsAg. Subsequently,
25 µg immunoglobulin is injected peritoneally once per day. Animals treated with
antibodies isolated from non-immunized rabbit serum die after about 60 days. This is
similar to untreated recipients of liver carcinoma cells. In contrast, mice treated with
30   antibodies isolated from immunized rabbit serum survive for more than 150 days. This

demonstrates that human antibodies produced in genetically engineered rabbits are capable of eliminating human carcinoma cells from SCID-mice.

What is claimed is:

1. An isolated nucleic acid molecule comprising the sequence as set forth in any one of SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, or SEQ ID NO: 13, or a portion of any one of SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, or SEQ ID NO: 13.

2. A recombination vector for replacing an Ig gene segment from a non-human animal with a human Ig gene segment, comprising from 5' to 3', a 5' nucleotide sequence, said human Ig gene segment, and a 3' nucleotide sequence, wherein said 5' nucleotide sequence and said 3' nucleotide sequence are homologous to the 5' and 3' flanking sequences of said Ig gene segment from the non-human animal.

3. The recombination vector of claim 2, wherein said non-human animal is an animal which relies primarily on gene conversion in generating antibody diversity.

4. The recombination vector of claim 3, wherein said animal is rabbit, pig, chicken, sheep or cow.

5. The recombination vector of claim 3, wherein the Ig gene segment from a non-human animal is a gene segment coding for a heavy chain or light chain constant region.

6. The recombination vector of claim 5, wherein said vector comprises from 5' to 3', a 5' nucleotide sequence as set forth in any one of SEQ ID NO: 12, SEQ ID NO: 13, a portion of SEQ ID NO: 12, or a portion of SEQ ID NO: 13; a human heavy chain constant region gene segment; a 3' nucleotide sequence as set forth in SEQ

ID NO: 10 or a portion of or SEQ ID NO: 10; and wherein said vector is useful for replacing a rabbit heavy chain constant region gene segment.

7. The recombination vector of claim 5, comprising the nucleotide sequence as set forth in SEQ ID NO: 51 wherein said vector is useful for replacing a rabbit heavy chain constant region gene segment.

8. The recombination vector of claim 5, wherein said vector is useful for replacing a rabbit light chain constant region gene and comprises a nucleotide sequence as set forth in SEQ ID NO: 53.

9. The recombination vector of claim 5, wherein said vector is useful for replacing a chicken light chain constant region gene and comprises a nucleotide sequence as set forth in SEQ ID NO: 57.

10. The recombination vector of claim 3, wherein the Ig gene segment from a non-human animal is a gene segment coding for a heavy chain or light chain variable region.

11. The recombination vector of claim 10, wherein said vector is useful for replacing a rabbit heavy chain variable region gene and comprises a nucleotide sequence as set forth in SEQ ID NO: 52.

12. The recombination vector of claim 10, wherein said vector is useful for replacing a rabbit light chain variable region gene and comprises a nucleotide sequence as set forth in SEQ ID NO: 54.

13. A transgenic vector comprising a humanized Ig locus, wherein said humanized Ig locus is derived from an Ig locus or a portion of an Ig locus of a non-human animal and comprises multiple Ig gene segments wherein at least one of said gene

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segments is a human Ig gene segment, wherein said gene segments are juxtaposed in an unrearranged, partially rearranged or fully rearranged configuration, and wherein said humanized Ig locus is capable of undergoing gene conversion and producing a repertoire of humanized immunoglobulins in said non-human animal.

14. The transgenic vector of claim 13, wherein said non-human animal is an animal which generates antibody diversity substantially by gene conversion.

15. The transgenic vector of claim 14, wherein said non-human animal is rabbit, pig, chicken, sheep or cow.

16. The transgenic vector of claim 13, wherein said humanized Ig locus is a heavy chain locus and comprises at least one V gene segment, at least one D gene segment, at least one J gene segment and at least one constant region gene segment.

17. The transgenic vector of claim 16, wherein said constant region gene segment is a human heavy chain constant region gene segment.

18. The transgenic vector of claim 17, wherein said human heavy chain constant region gene segment is a $C\gamma$.

19. The transgenic vector of claim 17, comprising about 10-100 V gene segments and at least one human V gene segment, wherein said human V gene segment is placed downstream to said 10-100 V gene segments.

20. The transgenic vector of claim 19, wherein said V gene segments are selected from V gene segments at the 3' V-region of said non-human animal and human V gene segments.

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21. The transgenic vector of claim 13, wherein said humanized Ig locus is a light chain locus and comprises at least one V gene segment, at least one J gene segment and at least one constant region gene segment.

5   22. The transgenic vector of claim 21, wherein said constant region gene segment is a human light chain constant region gene segment.

23. The transgenic vector of claim 22, wherein said human light chain constant region gene segment is Cλ or Cκ.

10

24. The transgenic vector of claim 22, comprising about 10-100 V gene segments and at least one human V gene segment, wherein said human V gene segment is placed downstream to said 10-100 V gene segments.

15   25. The transgenic vector of claim 24, wherein said V gene segments are selected from V gene segments at the 3' V-region of said non-human animal and human V gene segments.

26. The transgenic vector of claim 22, wherein said human V gene segment is placed

20   immediately 5' to a J gene segment in a rearranged configuration.

27. A method of making a transgenic vector comprising a humanized Ig locus capable of producing a functional repertoire of humanized antibodies in a non-human animal, comprising:

25   (i)   obtaining a DNA fragment comprising an Ig locus or a portion thereof from said non-human animal which comprises at least one V gene segment, at least one J gene segment and at least one constant region gene segment; and

           (ii)   integrating at least one human Ig gene segment into said DNA fragment

30   of step (i) to produce a humanized Ig locus, wherein said human Ig

53

gene segment is linked to the sequences of non-human origin operably
as to permit gene rearrangement and gene conversion of said
humanized Ig locus and the production of a functional repertoire of
humanized antibodies in said non-human animal.

28. The method of claim 27, wherein the integration of said human Ig gene segment is
achieved by homologous recombination, thereby replacing an Ig gene segment in
said Ig locus or said portion thereof from said non-human animal.

29. The method of claim 28, wherein the homologous recombination is achieved in a
bacterial cell, a yeast cell, or a non-human animal cell.

30. The method of claim 28, wherein the human Ig gene segment is provided on a
recombination vector, and is linked to a 5' nucleotide sequence and a 3' nucleotide
sequence which are homologous to the 5' and 3' flanking sequences of said Ig
gene segment from the non-human animal.

31. A transgenic animal comprising a humanized Ig locus, wherein said humanized Ig
locus is derived from an Ig locus or a portion of an Ig locus of a non-human animal
and comprises multiple Ig gene segments wherein at least one of said gene
segments is a human Ig gene segment, said gene segments being juxtaposed in an
unrearranged, partially rearranged or fully rearranged configuration, and wherein
said humanized Ig locus is capable of undergoing gene conversion and producing a
repertoire of humanized immunoglobulins in said non-human animal.

32. The transgenic animal of claim 31, wherein said animal is selected from rabbit,
pig, chicken, sheep or cow.

33. A B cell from the transgenic animal of claim 31.

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34. A method of making a transgenic non-human animal capable of producing a
    functional repertoire of humanized Ig heavy chains, comprising:

    (i)   introducing a transgenic construct according to any one of claims 16-20
5   into a recipient cell of a non-human animal and integrating the
             humanized heavy chain locus in the transgenic construct into the
             genome of said recipient cell; and

    (ii)   deriving an animal from the recipient cell having the humanized heavy
             chain locus integrated in the genome, thereby producing a functional
10   repertoire of humanized Ig heavy chains.

35. The method of claim 34, wherein said animal is rabbit and said recipient cell is a
    cell in an early embryo.

15   36. The method of claim 35, wherein said rabbit has an impaired expression of
    endogenous Ig molecules.

37. The method of claim 34, wherein said animal is chicken and said recipient cell is a
    fertilized egg.

20

38. The method of claim 37, wherein said chicken has an impaired expression of
    endogenous Ig molecules.

39. A method of making a transgenic non-human animal capable of producing a
25   functional repertoire of humanized Ig light chains, comprising:

    (i)   introducing a transgenic construct according to any one of claims 21-26
             into a recipient cell of a non-human animal and integrating the
             humanized light chain locus in the transgenic construct into the genome
             of said non-human animal; and

(ii)   deriving an animal from the recipient cell having the humanized light
        locus integrated in the genome, thereby producing a functional
        repertoire of humanized Ig light chains.

5   40. The method of claim 39, wherein said animal is rabbit and said recipient cell is a
        cell in an early embryo.

    41. The method of claim 40, wherein said rabbit has an impaired expression of
        endogenous Ig molecules.

10

    42. The method of claim 39, wherein said animal is chicken and said recipient cell is a
        fertilized egg.

    43. The method of claim 42, wherein said chicken has an impaired expression of
15   endogenous Ig molecules.

    44. A method of making a transgenic non-human animal capable of producing a
        functional repertoire of humanized antibodies, comprising:
        (i)   introducing a transgenic construct according to any one of claims 16-20
20   and a transgenic construct according to any one of claims 21-26 into a
               recipient cell of a non-human animal, and integrating the humanized Ig
               loci in the transgenes into the genome of said non-human animal; and
        (ii)   deriving an animal from the recipient cell having the humanized Ig loci
               integrated in the genome, thereby producing a functional repertoire of
25   humanized antibodies.

    45. A method of making a transgenic non-human animal capable of producing a
        functional repertoire of humanized antibodies, comprising
        (i)   making a transgenic non-human animal capable of producing a
30   functional repertoire of humanized heavy chains;

(ii)   making a transgenic non-human animal capable of producing a
        functional repertoire of humanized light chains; and

(iii)   mating the transgenic non-human animal of (i) with the transgenic
        animal of (ii); and

(iv)   selecting an offspring which produces both humanized heavy chains
        and humanized light chains thereby obtaining a transgenic non-human
        animal capable of producing a functional repertoire of humanized
        antibodies.

46. A humanized immunoglobulin produced using the transgenic animal of claim 31.

47. A humanized immunoglobulin derived from a transgenic animal, comprising at
least a portion of a human imunglobulin polypeptide sequence.

48. The humanized immunoglobulin of claim 47, wherein said transgenic animal
generates antibody diversity by gene conversion and/or hypermutation

49. The humanized immunoglobulin of claim 48, wherein said transgenic animal is a
rabbit, chicken, sheep or cow.

50. The humanized immunoglobulin of claim 49, wherein said human immunglobulin
polypeptide sequence is a heavy chain or light chain polypeptide sequence.

51. The humanize immunoglobulin of claim 50, wherein said portion of a human
immunglobulin polypeptide sequence is a human constant region polypeptide
sequence.

52. The humanized immunoglobulin of claim 51, wherein said human constant region
polypeptide sequence is Cγ, Cκ, or Cλ.

53. The humanized immunoglobulin of claim 51, wherein said portion of a human
immunoglobulin polypeptide sequence further comprising a human V domain
polypeptide sequence.

5   54. The humanized immunoglobulin of claim 47, wherein said humanized
immunoglobulin is specific for an antigen.

55. The humanized immunoglobulin of claim 54, wherein said antigen is a
microorganism selected from bacterium, fungus, or virus; an antigenic portion of
10   said organism; an antigenic molecule derived from said microorganism; or a
tumor-associated antigen.

56. The humanized immunoglobulin of claim 55, wherein said bacterim is selected
from *S. aureus*, *Pseudomonas aeruginosa*, enterococcus, enterobacter, or
15   *Klebsiella pneumoniae*.

57. The humanized immunoglobulin of claim 55, wherein said fungus is selected from
Candida albicans, Candida parapsilosis, Candida tropicalis, or Cryptococcus
neoformans.

20

58. The humanized immunoglobulin of claim 55, wherein said virus is selected from
respiratory synctial virus (RSV), Hepatitis C virus (HCV), Hepatits B virus
(HBV), cytomegalovirus (CMV), EBV, or HSV.

25   59. The humanized immunoglobulin of claim 55, wherein said antigen is selected from
Her-2-neu antigen, CD20, CD22, CD53, prostate specific membrane antigen
(PMSA), or17-1A molecule.

60. An antibody preparation, comprising the humanized immunoglobulin of any one
30   of claims 46-55.

61. The antibody preparation of claim 60, wherein said preparation is a monoclonal antibody preparation.

5   62. The antibody preparation of claim 60, wherein said preparation is a polyclonal antibody preparation.

63. The antibody preparation of claim 62, wherein said preparation is substantially non-immunogenic to human.

10

64. A pharmaceutical composition, comprising a pharmaceutically acceptable carrier and the antibody preparation of claim 60.

65. A method of treating a disease in a human subject comprising administering to

15   said subject a thereapeutically effective amount of the antibody preparation of claim 60.

66. The method of claim 59, wherein said disease is caused by bacterial, fungal or viral infection, or said disease is a cancer.

20

Figure 1(a)-(d). Novel nucleotide sequences 3'prime of the cow Cgamma gene
(Cow Cγ 3' flanking sequences). Primers are shown in shaded boxes. The 5'
primer is in CH3, and the 3' primer is in M1. The sequences of clone 11,
clone 3, and clone 5 are set forth in SEQ ID NO: 3, SEQ ID NO: 4 and SEQ ID
NO: 5, respectively.

```
         1                                                    50
clone11  CCTAC ACGTGTGTGG TGATGCACGA AACTTTACGG
clone3   CCTAC ACGTGTGTGG TGATGCACGA GGCCCTGCAC
clone5   CCTAC ACGTGTGTGG TGATGCACGA GGCCCTGCAC


         151                                                  200
clone11  AATCACTACA AAGAGAAGTC CACCTCGAGG TCTCCGGGTA AATGAGCCTC
clone3   AATCACTACA CGCAGAAGTC CACCTCTAAG TCTGCGGGTA AATGAGCCTC
clone5   AATCACTACA CGCAGAAGTC CACCTCTAAG TCTGCGGGTA AATGAGCCTC


         201                                                  250
clone11  GCGCCGCTGA TCTAGTGGAC GTTCCCTCAT CCACCCACCC CTCCCCCCAC
clone3   ACGTCCCTGC ACCAGCAAGC CCTCACCCAG C......... ..CCACCCTC
clone5   ACGTCCCTGC ACCAGCAAGC CCTCACCCAG C......... ..CCACCCTC


         251                                                  300
clone11  CCCGGGCTCC AGGTCCAGCC AGGGCGCCCT AGCCCCTCCC TGTGTGCATT
clone3   CCCGGGCTCC AAGTCCAGCC AGGACGCCCT AGCCCCTCCC TGTGTGCATT
clone5   CCCGGGCTCC AGGTCCAGCC AGGACGCCCT AGCCCCTCCC TGTGTGCATT


         301                                                  350
clone11  CCTCCTGGGC CGCCGTGAAT AAAGCACCCA GGCCGCCCTG GGACCCTGCA
clone3   CCTCCTGGGC CGCCGTGAAT AAAGCACCCA GGCCACCCTG GGACCCTGCA
clone5   CCTCCTGGGC CGCCGTGAAT AAAGCACCCA GGCCGCCCTG GGACCCTGCA


         351                                                  400
clone11  ACGCTGTGCT GGTTCTTTCC GAGGCAGAGC CCTGGTGGCC GCCAGGCCTG
clone3   ACGCTGTGCT GGTTCTTTCC GAGGCAGAGC CCTGGTGGCC GCCAGGCCTG
clone5   ACGCTGTGCT GGTTCTTTCC GAGGCAGAGC CCTGGTGGCC GCCAGGCCTG


         401                                                  450
clone11  CGGGGGTGGG CTGAGCCGAC TCTGGGCCAC TTTGTTCAGC ATCTGTGGGG
clone3   CAGGGGTGGG CTGAGCCGAC TCTGGGCCAC TTTGTTCAGC ATCTGTGGGG
clone5   CGGGGGTGGG CTGAGCCGAC TCTGGGCCAC TTTGTTCAGC ATCTGTGGGG


         451                                                  500
clone11  GAGCTGACCC CACTCCGGGC CAGACACACA GTGAGTGGGT CCAGCAGGCC
clone3   GAGCTGACCC CGCTCCGGGC CAGACACACA GTGAGTGGGT CCAGCAGGCC
clone5   GAGCTGACCC CACTCCGGGC CAGACACACA GTGAGTGGGT CCAGCAGGCC


         501                                                  550
clone11  ACCTGGGGGC TGCCCAAGGC CACAGAGGGG CTTGGCCAGA GGCACAGCTC
clone3   ACCTGGGGGC TGCCCGAGGC CACGGAGGGG CTTGGCCAGA GGCGTACCTC
clone5   ACCTGGGGGC TGCCCAAGGC CACAGAGGGG CTTGGCCAGA GGCACAGCTC
```

Figure 1(a)

```
           551                                                600
clone11  CACGGTCCCC TCCAGCCACC ACCTGCTGGG CCGGCCTCTG GACAGGAACC
clone3   CACGGCCCCC TCCAGCCACC ACCTGCTGGG CCGGCCTCTG GACAGGAACC
clone5   CACGGTCCCC TCCAGCCACC ACCTGCTGGG CCGGCCTCTG GACAGGAACC

           601                                                650
clone11  GGGGAAGCCC CCGAGACCCT CAGGGATTGA GGCCCAATGC TTCCCGCCTC
clone3   GGGGAAGCCC CCGAGACCCT CAGGGATTGA GGCCCAATGC TTCCCGCCTC
clone5   GGGGAAGCCC CCGAGACCCT CAGGGATTGA GGCCCAATGC TTCCCGCCTC

           651                                                700
clone11  TGCTCCAGCC CACGCTGTGG GGCAGGGCCA CATCCTTGTC CCCAGGCCCC
clone3   TGCTCCAGCC CACGCTGTGG GGCAGGGCCA CATCCTTGTC CCCAGGCCCC
clone5   TGCTCCAGCC CACGCTGTGG GGCAGGGCCA CATCCTTGTC CCCAGGCCCC

           701                                                750
clone11  TGTCCTTGGG TGTCCAGAGT CCTTGTGTCC ACTCTGGGCC TGCCTGGAGC
clone3   TGTCCCTGGG TGCCCAGAGT CCTTGTGTCC ACTCTGGGCC TGCCTGGAGC
clone5   TGTCCTTGGG TGTCCAGAGT CCTTGTGTCC ACTCTGGGCC TGCCTGGAGC

           751                                                800
clone11  CACGCATGGC CAGGGGGTGG CCCTGCTTCA CCCTCAGGCT CCCAAGGTCA
clone3   CACGCGTGGC CGGGGGATGG CCCTGCTCCA CCCTCAGGCT CCCAAGGTCA
clone5   CACGCATGGC CAGGGGGTGG CCCTGCTTCA CCCTCAGGCT CCCAAGGTCA

           801                                                850
clone11  GGCCTCGCCC TCCCTCGGCC AGGAGGCTCT GCCCGGCTCT CCCTGCCCAG
clone3   GGCCTCGCCC TCCCTCAGCC AGGAGGCTCT GCCCGGCTCT CCCTGCCCAG
clone5   GGCCTCGCCC TCCCTCGGCC AGGAGGCTCT GCCCGGCTCT CCCTGCCCAG

           851                                                900
clone11  GGCCAGGCCT GTGCGCCCAT GGGGAGGTCA TCCCTGTGCC TGAAAGGGGT
clone3   GGCCAGGCCT GTGCGCCCAT GGGGAGGTCA TCCCTGTGCC TGAAAGGGGCT
clone5   GGCCAGGCCT GTGCGCCCAT GGGGAGGTCA TCCCTGTGCC TGAAAGGGGT

           901                                                950
clone11  CCAGGCCGAG AGCCCTGAAT GTCCAGGGCA GGGACCTAGC TGCTCCCTGT
clone3   CCAGGCCGGG AGCCCTGAAT GTCCAGGGCA GGGACCTAGC TGCTCCCTGC
clone5   CCAGGCCGAG AGCCCTGAAT GTCCAGGGCA GGGACCTAGC TGCTCCCTGT

           951                                               1000
clone11  GGACACGGAG CCCAGAGCCA CAGACAACAA GCCCCAGCCC CGCACGCACA
clone3   AGACACGGAG CCCAGAGCCA CAGACAACAA GCCCCAGCCC CGCACGCACA
clone5   GGACACGGAG CCCAGAGCCA CAGACAACAA GCCCCAGCCC CGCACGCACA

           1001                                              1050
clone11  CGAGACAGCC CGCACCCAGC CTCCTCCACA CGCACTCAGG TGTACATGCG
clone3   CAAGACAGCC CGCACCCAGC CTCCTCCACA CGCACTCAGG TGTGCATCCG
clone5   CGAGACAGCC CACACCCCGC CTCCTCCACA CGCACTCAGG TGTGCATCCG
```

Figure 1(b)

```
          1051                                                      1100
clone11   CACATGAGCA CACTTCACCC CGTCACACCC ACACACCTAC ACACACTCAG
clone3    ·CACATGAGCA CACTTCACCC ·CGTCACACCC ACACGCCTAC ACACACTCAG
clone5    CACATGAGCA CACTTCACCC CATCACACCC ACACGCCTAC ACACACTCAG

          1101                      .                              1150
clone11   GTCTCGCACT ·CGGGGACCCA TGGGGTGACC CCACGGGCCC AGA.CCAGAG
clone3    GTCTCGCACT CGGGGACCCA TGGGGTGACC CCACAGGCCC AGACCCAGAG
clone5    GTCTCGCACT CGGGGACCCA TGGGGTGACC CCACAGGCCC AGACCCAGAG

          1151                                                      1200
clone11   CTGGGTCTTG TGAGCCCTCC CTGTGGACAC CAGCTGGGCC CCACCCTCCA.
clone3    CTGGGTCTTG TGAGCCCTCC CTGTGGACAC CAGCTGGTCC CCACCCTCCA
clone5    CTGGGTCTTG TGAGCCCTCC CTGTGGACAC CAGCTGGTCC CCACCCTCCA

          1201                                                      1250
clone11   GCGCCCATGG GCTGCTCAGC GGCCCTTTCC CACACTGACC ACACTGACCA
clone3    GCGCCCATGG GCTGCTCAGT GGCCCTTTCC CACACTGACC ACACTGACCA
clone5    GCGCCCGTGG GCTGCTCAGC GGTCCTTTCC CACACTGACC ACACTGACCA

          1251                                                      1300
clone11   GGTCAGACAT CCGTTCCTTG CCTCCCCTGG GACACCCACG CCCCTCCCTA
.clone3   GGTCAGACAT CCGTTCCTTG CCTCCCCTGG GGCACCCACG CCCCTCCCTA
clone5    GGTCAGACAT CCGTTCCTTG CCTCCCCTGG GGCACCCATG ·CCCCTCCCTA

          1301                                                      1350
clone11   GCAGGCTGAG ATCCCCCCTC AGCCCCTCGT CCTGGCAGCC TCACCCCTCG
clone3    GCAGGCTGAG ATCCCCCCTC AGCCCCTCGT CCTGGCACCC. TCACCCCTCA
clone5    GCAGGCTGAG ATCCCCCCTC AGCCCCTCGT ·CCTGGCACCC TCACCCCTCA

          1351                                                      1400
.clone11  GGCACAGCAC CCCTCAGGCC CGGTGCTGTC AGCCCTCCCT CCCCGGGGGC
 clone3   GGCACAGGGA CAC...AGCC CGGCGCTGTC TGCCCTCCCT CCCTGGGGGC
 clone5   GGCACAGGGA CAC...AGCC CGGTGCTGTC TGCCCTCCCT CCCTGGGGGC

          1401                          -                          1450
clone11   AGGGCCCAGG AACGTGCGCT CTGCTGACCC TCCCAGCTCC AGGCCTGGCC
clone3    AGGGCCCAGG CTCACATGCT CTGCTGACCC TCCCGGCTCC AGGCCTGGCC
clone5    AGGGCCCAGG CTCACATGCT CTGCTGACCC TCCCAGCTCC AGGCCTGGCC

          1451                                                      1500
clone11   CCCAGGGCAG AGGAGGCCAG GAACTGAGCC ·TCTGTCCTGT GGGGAGGTAG
clone3    ·CCCAGGGCAG AGGAGGCCAG GAACTGAGCC TCTGTCCTGG GGGGAGGTGG
clone5    CCCAGGGCAG AGGAGGCCAG GAACTGAGCC TCTGTCCTGG GGGGAGGTGG

          1501                                  .                  1550
clone11   GGTCAGGGTC CCAGCTCAGG GCACAGCTCA GGATGGGAGC AGGACCCCAC
clone3    GGTCAGGGCC CCAGCTCAGG GCACAGCTCA GGATGGGAAC AGGACACCAC
clone5    GGTCAGGGCC CCAGCTCAGG GCACAGCTCA ·GGATGGGAGC AGGACACCAC
```

Figure· 1(c)

```
       1551                                                          1600
clone11 AGGCCAGGCC CAGATAGCAG CCAGGGCTGG AGGGGTTGGG GCTGGGGCTG
clone3  AGGCCAGGCC CAGACAGTGG CCAGGGCTGG AGGGGTGGGG TCTGGGGCTG
clone5  AGGCCAGGCC CAGACAGTGG CCAGGGCTGG AGGGGTGGGG TCTGGGGCTG

       1601                                                          1650
clone11 GGCCCCAGAG ACTGACCTCA GGTGACCCCT GCCTGGCCCA TGGGGAGATC
clone3  GGGCCCAGAG ACTGACCTCA GGTGATCCCT GCCCAGCCCA TGGGGGGATC
clone5  GGCCCCAGAG AATGACCTCA GGTGATCCCT GCCCAGCCCA TGGGGGGATC

       1651                                                          1700
clone11 ACGCCACCTT CCCCCCACCC AGAGGGAGCC CTGCCC...T ACCCCAGTGA
clone3  CTGCCACCTT CCCCCCACCC AGAGGGAGCC CTGCCCCGAG GCCCTGATGA
clone5  CTGCCACCTT CCCCCCACCC AGAGGGAGCC CTGCCCCGAG GCCCTGATGA

       1701                                                          1750
clone11 CCCTGCCCAG CCCTCCGTGG GCAGACACAG CACTGACCAC CCCTCCCTGT
clone3  TGCCACCCAG CCCCCCGTGG GCAGACACAG CACTGACCAC CCCTCCCTGT
clone5  TGCCACCCAG CCCCCCGTGG GCAGACACAG CACTGACCAC CCCTCCCTGT

       1751                                                          1800
clone11 GCAGACTTGC TGCTGGAGGA GGAGATCTGT GCGGACGACC TGGATGGGGA
clone3  GCAGACCTGC TGCTGGAGGA GGAGATCTGT GCGGACGCCC AGGACGGGGA
clone5  GCAGACCTGC TGCTGGAGGA GGAGATCTGT GCGGACGCCC AGGACGGGGA

       1801                                                          1850
clone11 GCTGGACGGG CTC▓GGACCA CGATCTGCAT GTT▓
clone3  GCTGGACGGG CTC▓GGACCA CGATCACCAT GTT▓
clone5  GCTGGACGGG CTC▓GCACCA CGATCACCAT GTT▓
```

Figure 1(d)

Figure 2(a)-(e). Novel nucleotide sequnces 3'prime of the sheep Cgamma
genes. Primers are shown in shaded boxes. The 5' primer is in CH3, and the
3' primer is in M2. The sequences of clone 11 and clone 1 are set forth in
SEQ ID NO: 8 and SEQ ID NO: 9, respectively.

```
        101                                                 150
clone11 CCTACGGCGTC TGTGGTGATG CACGAGGCTC TACACAACCA CTACACACAG
clone1  CCTACGGCGTC TGTGGTGATG CACGAGGCTC TGCACAACCA CTACACACAG

        151                                                 200
clone11 AAGTCGATCT CTAAGCCTCC GGGTAAATGA GCCACATGCC CCCGCACCAG
clone1  AAGTCGGTCT CTAAGCCTCC GGGTAAATGA GCCACACGCC CCCGCACCAG

        201                                                 250
clone11 CAAGCCCTCA CCCAGCCCGC CCTCCCCGGG CTCCAGGTCC AGCCAGGACG
clone1  CAAGCCCTCA CCCAGCCCGC CCTCCCCGGG CTCCAGGTCC AGCCAGGACG

        251                                                 300
clone11 CCCTAGCCCC TCCCTGTGTG CATGCCTCCT GGGCCGCCAT GAATAAAGCA
clone1  CCCTAGCCCC TCCCTGTGTG CATGCCTCCT GGGCCGCCAT GAATAAAGCA

        301                                                 350
clone11 CCCAGGCCGC CCTGGGACCC TGCAACGCTG TGCTTGTTCT TTCCGAGGCA
clone1  CCCAGGCCGC CCTGGGACCC TGCAGCGCTG TGCTGGTTCT TTCCGAGGCA

        351                                                 400
clone11 GAGCCCTGGT GACCGCCAGG CCTGCGGGGG GTGGGCTGAG CCCACTCTGG
clone1  GAGCCCTGGT GATCGCCAGG CCTGCGGGGG GCGGGCTGAG CCCACTCTGG

        401                                                 450
clone11 GCCGCTTGGT TCAGCATCTG TGGGGGCGCT GACCCCTCTC CGGGCCAGAC
clone1  GCCGCTTGGT TCAGCATCTG TGGGGGCGCT GACCCCTCTC CGGGCCAGAC

        451                                                 500
clone11 ACACAGTGAG TGGGTCCGGC AGGGCACCTG GGGGCTGCCC GAGGCCTCGG
clone1  ACACAGTGAG TGGGTCCGGC AGGGCACCTG GGGGCTGCCC GAGGCCTCGG

        501                                                 550
clone11 AGGGGCTTGG CCAGAGGCGC AGCTTCACGG CCCCCTCCAG CCACCACATT
clone1  AGGGGCTTGG CCAGAGGCGC AGCTCCACGG CCCCCTCCAG CCACCACATT

        551                                                 600
clone11 CTGGGCCAGA CTCTGGGCAG GAACGGGGGA AGCCCCCGAC ACCTCAGGGA
clone1  CTGGGCCAGA CTCTGGGCAG GAACGGGGGA AGCCCCCGAC ACCTCAGGGA

        601                                                 650
clone11 TTGAGGCCCA ACGCTTCCCG CCTCTGCTCC AGCCCACGCT GAGGGGCAGG
clone1  TTGAGGCCCA ACGCTTCCCG CCTCTGCTCC AGCCCACGCT GAGGGGCAGG
```

Figure 2(a)

```
           651                                                    700
clone11  GCCGCGGCCT TGTCCCCAGG CCCCTGTTCC TGGGTGCCCA GAGTCCGTGT
clone1   GCCGCGGCCT TGTCCCCAGG CCCCTGTTCC TGGGTGCCCA GAGTCCGTGT

           701                                                    750
clone11  GTCCACTCTG GGCCTGCCTG GAGCCAGACT GGCCCAGGGG GAGGCCCTGC
clone1   GTCCACTCTG GGCCTGCCTG GAGCCAGACT GGCCCAGGGG GAGGCCCTGC

           751                                                    800
clone11  TTCACCCTCA GGCTCCCGAG GTCAGGCATC ATCCTCGTCG GCCAGTAGCT
clone1   TTCACCCTCA GGCTCCCGAG GTCAGGCATC ATCCTCGTCG GCCAGTAGCT

           801                                                    850
clone11  CTGCCTGGCT CTCTCTGCCC GGGGCCAAGC CTGTGTGCCC ATGGGGAGGT
clone1   CTGCCTGGCT CTCTCTGCCC GGGGCCAAGC CTGTGTGCCC ATGGGGAGGT

           851                                                    900
clone11  CGTCCCTGTG CCTGAAAAGG GCCCAGGCTG GGAGCCCTGA ACGTCCAGGG
clone1   CGTCCCTGTG CCTGAAAAGG GCCCAGGCTG GGAGCCCTGA ACGTCCAGGG

           901                                                    950
clone11  CAGGGACCTA GCTGCTCCCT GGGGACACTG AGCCCAGAGC CCCAGACACC
clone1   CAGGGACCTA GCTGCTCCCT GGGGACACTG AGCCCAGAGC CCCAGACACC

           951                                                    1000
clone11  AAGCCCCAGC CCCGCACGCA CACGAGACAG CCCACACCCA GCGTCCTCCA
clone1   AAGCCCCAGC CCCGCACGCA CACGAGACAG CCCACACCCA GCGTCCTCCA

           1001                                                   1050
clone11  CACGCACTCA GGCGTCCACC CGCACACAAG CATGCTTCAC CCCCGTCACA
clone1   CACCCACTCA GGCGTCCACC CGCACACAAG CATGCTTCAC CCCCGTCACA

           1051                                                   1100
clone11  CACCCACATG CCTGCACACA CTCAGGTCTC ACGCTCCGGG ACCCATGGAG
clone1   CACCCACATG CCTGCACACA CTCAGGTCTC ACGCTCCGGG ACCCATGGAG

           1101                                                   1150
clone11  TGATCCCACG GGCCCAGACC CAGAGCTGGG TCTCATGAGC CCTCCCTGTG
clone1   TGATCCCACG GGCCCAGACC CAGAGCTGGG TCTCATGAGC CCTCCCTGTG

           1151                                                   1200
clone11  GACACCAGCT GGTCCCCATT CTCCAGCGCC CTTGGGCTGC TCAGTGGCCC
clone1   GACACCAGCT GGTCCCCATC CTCCAGCGCC CTTGGGCTGC TCAGTGGCCC

           1201                                                   1250
clone11  TTTCCCACAC TGACCACACT GACCAGGTCA GACATCCTTC CTCGCCTCCC
clone1   TTTCCCACAC TGACCACACT GACCAGGTCA GACATCCTTC CTCGCCTCCC
```

Figure 2(b)

```
       1251                                      1300
clone11 CTGGGGCACC CACGCCCCTC CCTCGCAGGC TGAGACCCCC CCTCAGCCCC
clone1  CTGGGGCACC CACGCCCCTC CCTTGCAGGC TGAGACCCCC .CCTCAGCCCC


       1301                                      1350
clone11 TCGTCCTGGC ACCCTCACCC CTCGGGCACA GGGACACAGC CCGGCACTGT
clone1  TCGTCCTGGC ACCCTCACCC CTCGGGCACA GGGACACAGC CCGGCACTGT


       1351                                      1400
clone11 CTGCCCTCCC TCTCGGGGAC AGAGCCCAGG CACGTGTGCT CTGCTGAGCC
clone1  CTGCCCTCCC TCTCGGGGAC AGAGCCCAGG CACGTGTGCT CTGCTGAGCC


       1401                                      1450
clone11 TCCCGGCTCC AGGCCTGGCC CCCAGGGCAG AGGAGGCCAG GAATTGAGCC
clone1  TCCCGGCTCC AGGCCTGGCC CCCAGGGCAG AGGAGGCCAG GAATTGAGCC


       1451                                      1500
clone11 TCTGTCCTGC GGGGAGGTGG GGTCAGGGCC CCAGCTCAGG GCACAGCTCA
clone1  TCTGTCCTGC GGGGAGGTGG GGTCAGGGCC CCAGCTCAGG GCACAGCTCA


       1501                                      1550
clone11 GGATGGGAGC AGGACCCCAC AGGCCAGGCC CAGACAGTGG CCAGGGCTGG
clone1  GGATGGGAGC AGGACCCCAC AGGCCAGGCC CAGACAGTGG CCAGGGCTGG


       1551                                      1600
clone11 GGCTGGGGCT GGGGCCCAGA GACTGACCTC AGGTGACCCC TGCCCGGCCC
clone1  GGCTGGGGCT GGGGCCCAGA GACTGACCTC AGGTGACCCC TGCCCGGCCC


       1601                                      1650
clone11 ATGGGGGATC ACACCGCCAT CCCCCCCGCC GCAGAGGGAG CCCTGCCCCG
clone1  ATGGGGGATC ACACCGCCAT CCCCCCCGCC GCAGAGGGAG CCCTGCCCCG


       1651                                      1700
clone11 AAGCCCCGAT GGCCCCGCCC AGCCCCCCGT GGGCAGACAC AGCACTGACC
clone1  AAGCCCCGAT GGCCCCGCCC AGCCCCCCGT GGGCAGACAC AGCACTGACC


       1701                                      1750
clone11 CCCCTCCCTG TGCAGATCTG CTGCTGGAGG AGGAGAGCTG TGCGGACGCC
clone1  CCCCTCCCTG TGCAGATCTG CTGCTGGAGG AGGAGAGCTG TGCGGACGCC


       1751                                      1800
clone11 CAGGACGGGG AGCTGGACGG GCTCTGGACG ACTATCTCCA TCTTCATCAC
clone1  CAGGACGGGG AGCTGGACGG GCTCTGGACG ACTATCTCCA TCTTCATCAC


       1801                                      1850
clone11 GCCCTTCCTG CTCAGCGTCT GCTACAGCGC CACCGTGACC CTCTTCAAGG
clone1  GCTCTTCCTG CTCAGCGTCT GCTACAGTGC CACCGTGACC CTCTTCAAGG
```

Figure 2(c)

```
            1851                                                      1900
clone11  TGGGGGTCCA CCCTGCTGGG CCCTCGGGCC CCCTCTCTGT CCCCAGGGTC
clone1   TGGGGGCCCA CCCTGCTGGG CCCTCGGGCC CCCTCTCTGT CCCCAGGGTC

            1901                                                      1950
clone11  CCCGCAGAGT CCCTCCCTGC CCCTCACTGT CCCTCCCTGT CCCTCTCTGT
clone1   CCCGCAGAGT CCCTCCCTGC CCCTCACTGT CCCTCCCTGT CCCTCTCTGT

            1951                                                      2000
clone11  CCCTCTCTGT CCCTCTCTGT CCCTCTCTGT CCGTTCATTT TCCCTTCACC
clone1   CCCTCTCTGT CCCTCTCTGT CCCTCTCTGT CCGCTCATTT TCCCTTCACC

            2001                                                      2050
clone11  GTAAGCTTGA GACAGATTGG GGTCATTTCA GAGGGCGTCT GAAGAGTCTC
clone1   GTAAGCTTGA GACAGATTGG GGTCATTTCA GAGGGCGTCT GAAGAGTCTC

            2051                                                      2100
clone11  TGTGCCGCAC GCCTCCCTTC ATGTCAGTGG GGAGAATTCA GCAAGGGTGG
clone1   TGTGCCGCAC GCCTCCCTTC ATGTCAGTGG GGAGAATTCA GCAAGGGTGG

            2101                                                      2150
clone11  AGTGCTGGGT GAGAAATGAG GCTTGCGGCG CTCACGAGCA GTGATGGGGC
clone1   AGTGCTGGGT GAGAAATGAG GCTTGCGGCG CTCACGAGCA GTGATGGGGC

            2151                                                      2200
clone11  ACTGCTGCTC CCTGAGACCT GCGCGGACAC CGTTTTCCAT CGCAGGAGAA
clone1   ACTGCTGCTC CCTGAGACCT GCGCGGACAC CGTTTTCCAT CGCAGGAGAA

            2201                                                      2250
clone11  GCGGGCAAGG GAAAACGCCC TCTTGGTCTC TCTTGAGTAA ATGTCGCGTT
clone1   GCGGGCAAGG GAAAACGCCC TCTTGGTCTC TCTTGAGTAA ATGTCGCGTT

            2251                                                      2300
clone11  TTGGTCATCA GTCCCTCCCC CAGTGAGGCT AGAGGAGTTT ACTTCTCCCT
clone1   TTGGTCATCA GTCCCTCCCC CAGTGAGGCT AGAGGAGTTT ACTTCTCCCT

            2301                                                      2350
clone11  CTCGATGGTC AGGTCAGGAC TGTCATAGAC TCCGGATCAC CTTCCTGTAA
clone1   CTCGATGGTC AGGTCAGGAC TGTCATAGAC TCCGGATCAC CTTCCTGTAA

            2351                                                      2400
clone11  ATGCTTGCTT TTTGTGTGCA GAGAGCCTGT TTTAGCTCGG GGGTCCTCAG
clone1   ATGCTTGCTT TTTGTGTGC. .AGAGCCTGT TTTAGCTCGG GGGTCCTCAG

            2401                                                      2450
clone11  CTCACTGAGC TCGCGGGGCA GGGGTGGGCT CGGGCTGGCG CCGCCTGTTC
clone1   CTCACTGAGC TCGCGGGGCA GGGGTGGGCT CGGGCTGGCG CCGCCTGTTC
```

Figure 2(d)

```
        2451                                                      2500
clone11 GGGAGCGCAT CTCCAGCATG CTGTCGCACA GCTTCGTTGC TAACAAGACC
clone1  GGGAGCGGCA TCTCCAGCTG CTGTCGCACA GCTTCGTTGC TAACAAGACC

        2501                                                      2550
clone11 GCTTAGTCTC GTGGTTAGAC CAACCTGCTT TCTCGAGTAA TTGTTAATTT
clone1  GCTTAGTCTC GTGGTTAGAC CAACCTGCTT TCTCGAGTAA TTGTTAATTT

        2551                                                      2600
clone11 ACAGGAGTTT CCTGTATTTT TCAACTTATA ATCCCCTAGT CAGATAACTC
clone1  ACAGGAGTTT CCTGTATTTT TCAACTTATA ATCCCCTAGT CAGATAACTC

        2601                                                      2650
clone11 TTTAATCACC TATTCTGCCC CTTCATTTTC TCCCTATCGA TCTCAGCAAC
clone1  TTTAATCACC TATTCTGCCC CTTCATTTTC TCCCTATCGA TCTCAGCAAC

        2651                                                      2700
clone11 CCATCACTGC CCTCACTGTC CTTAAACTGT CCCTTAACTG ACCAGACTGT
clone1  CCATCACTGC CCTCACTGTC CTTAAACTGT CCCTTAACTG ACCAGACTGT

        2701                                                      2750
clone11 CCCTCAGTGT CCCCTCAGAG TCACCTCCCT ATCACCTCAC TGTCCCTCTC
clone1  CCCTCAGTGT CCCCTCAGAG TCACCTCCCT ATCACCTCAC TGTCCCTCTC

        2751                                                      2800
clone11 TGCCCCTCTC TGCCCCTCTC TGTCCCTCCC TGCCCCTCCC CGTCCCCTCT
clone1  TGCCCCTCTC TGCCCCTCTC TGTCCCTCTC TGTCCCTCCC CGTCCCCTCT

        2801                                                      2850
clone11 CTGTCCCTCT CTGCCCCTCA CTGCTCCTCT CTGCACCTCA CTGCTCCTCA
clone1  CTGTCCCTCT CTGCCCCTCA CTGCTCCTCT CTGCACCTCA CTGCTCCTCA

        2851                                                      2900
clone11 CTGCCCTGGG GGAGGCCCGC ATCGAGGTGT CTCTGCTCAC CCCGTCCCCC
clone1  CTGCCCTGGG GGAGGCCCGC ATCGAGGTGT CTCTGCTCAC CCCGTCCCCC

        2901                                                      2950
clone11 ACCCCGTACC CCCCGCCAGG TGAAGTGGAT CTTCTCCTCGTCT
clone1  ACCCCGTCCC CCCCGCCAGG TGAAGTGGAT CTTCTGGTCGTCT
```

Figure 2(e)

Figure 3(a)-(b). Novel 3'prime flanking sequence (SEQ ID NO: 10) of rabbit
Cgamma gene.

CTCCCCGCCACGCCGCCAGC

```
TGTGCACCCCGCACACAAATAAAGCACCCAGCTCTGCCCTGAGAGGCTGTCCTGATTCCT
TCCAAGGCAGAGGCTTCCACTCGGGCCGGACAGGGTTGGGCGGGCGCCGTGGGCTCTGCT
GTGGCCAGCAGCCAGAACGGTCAACAGTGGGACAGGGGCAGACCCACAGCACAGGGGCCT
GCCAAGAACTGGGCTCAGCCGGAGTGCTGTGGCAGGTCCCCCCTTGCAGCTAGCACGTGT
GTGCTGGGCAGGCAGAGGCCCCAGGGGAGGAGCACACAGCTACCACCTCTGCAAGAGCC
TGGCCTGGCGCCCAGGTCCCAGTCCACAGGGTGTGTAGTACACAGAGCCTCATCTTACCA
CAGATGTAGGGACAGACCCACCACGCCCCTGCACCCCACCCAGCCTCGCCCCTTGTGGGA
CCAGGGCTACCACTCCACTCCCCCGCCCAGAGCAGCAGAAGCAGGTGGCATCCTCAGCAG
AGGGACAGTCTCACCCCTCCACGGCACTGAGCCCTGACCCATCAAACAAGCCCCTCCTGC
TGCACAGCACCTGTGTGCACATCACACACACACACACACACACTGAGGCCTGACCCCCA
TCAAACAAGCCCCTCCTGCTGCACAGCACCTGTGTGCACATCACACACACACACACACAC
ACACTGAGGCCTGACCCCATCCTGCCCTCCTGCTGCATGGCACCTGTGTGCACATCACAC
ACACATGCACACACACACTCACACACACTGAGCCCTGACCCCATCCTGCCCTCCTGCTGC
ATGGCACCTGTGTGCACATCACACACACACACACACACACACACACACTGAGCCCTGACC
CCATCCTGCCCTCCTGCTGCATGGCACCTGTGCACACATCACAAACACGCCTGCCTCATA
CACTGGCACTCAGAAGGGGCCCCTGTACACGCATACACATGCACACACCTTGACACATGG
GCCCCCTACACACGCATCACACACACTCATGCACACTCCTCACACATGGCCCTCCTGCAC
ATACATTGCACACACATGTGCACAGACCTCACAATGGGCCCCTGCACACACATTGTACAC
ACGCATGTGCACACACTTCACACATGGGCCCCCTGCACATGCATTGCACACACAGACACA
CACATGTGCATTCCTCACACATTGGGGCCTTGCAAGGGATGCCCTGCACACACATTGCAC
ATGCTCACATGTGCACACACCCCACACTGGAGCCTTGCATAGGGCCCCCTGTACACACAC
CATGCATACACACACACCTCACACAAGGGGCCCCCTACATACGCAAAACACACACACACA
CATGCACACACCTCATACACGGGCCCCCCTACACACATCACACACACACACACACACACGT
ATGCATGCCTCACACACAGACCTTGCAAGGGGCCCCCTGCACATGCATCAAACACATATG
CACATGTTTCACACACACGGTCCCCTACACACACTGCACACGCACACATGTGTACATGCT
TCACACACTGGGGCCTTGCATGGGGTCCCCTGCATAGCATAGCACCCAGAGCCACGCCAG
GTGCCTGGGCACATGGACACTGGTGCACACACAGCACCCAAGCCCAGCTCTCCCATCCAA
GGGGCACCAGCACCCCCCACTCACGAGCACCCTGAATTCCTGCTCCCCACAAGCGAACGT
GCACCCTACCTCTCCAGACGTCCCTTTCCTGTGGCCACTCCCATAGGTATTGGCGAGACC
CTCCCTTGACCCTTGGGCCTGGTCACCCAGGGGACAGGAGAGGGCCAAGTTGGGCCACAG
TACCACTGCCCAGCAGGGGTGAGGCAAGCAGAGGGTGGGTCTGTGAGGCGTCTGGCCAGC
CGTGCTGGGGCCCAGGTGGGGAGCAGCTGGGTGGCTGAGGTGGCTTCCTTGCAGGTGGTT
GGGGGGGAGCTGGCCCCACAAGTGCCACTGCCCAGCACTGTCCAGTGCTTCCCCCTGAACC
TCCCGGCCACCCATCCCCAGCTGCAGCCGCAGAGGGAGTGCCCCTCGGCCTCCTCGGCAA
GACGCACGCTGACTGCCCCTCCCCATCCAGAGCTGCAGCTGGACGAGAGCTGTGCCGAGG
CCCAGGACGGGGAGCTGGACGGGCTGTGGACCACCATCACCATCTTCATCTCCCTCTTCC
TGCTCAGCGTGTGCTACAGCGCCACAGTCACCCTCTTCAAGGTGGGTGCTGCACCCGGCA
CGGGTGGGCTGGGGGCCAGGGGCGGGGGCCGGGGGCCAGGCCCTCCTCACCCCGCGCCGC
CGCTGCTGCAGGTGAAGTGGATCTTCTCGTCCGTGGTGGAGCTGAAACACACCATCGCTC
CCGACTACAGGAACATGATCGGGCAGGGGGCCTAGGCCCTTCGTTCTCACAGCCTGCCTC
CCTGGCCAGCAGGAGCCCCCGCCTCCGCCTCGGACCCCATGGCTCTCTGCTCTGGCCGCT
CCGGACCCCTCCGCCTCGGGAGAAGCGCGCAGCTGATGCCTGCCGGCCCCTCCACGCAGC
AGTGCGGACAGCACGCATCTGTCGTCCACCCGGCAGGACCCCACCCAGGGCCAGCCCTGA
CCGCCAGCCTCCTGGACTCAGGGCTCCTCTGAGAAAAGGCCCACTTGTTGGTCCCCTCAG
CCCACACCCAGGCAGCCTCCGGTGGGTGCTTCCCTGGACCCCAGCCTGAGGCCTATGCTT
GTTCTCCTGTGGCTCTTACTCAGAGGCCCGTGCTGGACTCCCACCCACAGGGACAGTGCC
```

Figure 3(a)

CTGCTCCAACCCTCACTGCACTGGGGGTCATGGGGCCACCTTCTGTGCAGGGGTCCTGGC
TCCAAGGAGAACACTCGAAGGGCCTGCTTGGCCACCTGGCACCACGGGAGCCCCGCTGGG
TAGCTTGGCAGGGACCCCTGAGTAGAGGTGGGTGCACCCAGCCAGAAAGCCTGCTGGATG
GACAGGAGCCTGGCGTCCGGGCCCCAGGCAGGCAGACACGGCTTCATGGACAGGAGAGGC
CAAGGAACATCAGCAAAGAGAGACAGCTGGGCCGGGCGTTCCAGCCAGACCCATCCTGCA
GCCCAGCATCGGCCGGGTGCTACTACAGCAGGCAGCAGGGTCT

Figure 3(b)

Figure 4. Novel nucleotide sequence (SEQ ID NO:11) 3'prime of the rabbit
Ckappa 1 gene.

```
GGCAGACGGCTGCGACGGCTAGCGCCAGTGACCCTGAG
GCCCAGCCTCGCCGCTCCCTCCCCTCAGTGGACCCATTCCCACCACAGTCCTCCAGCCCC
TCCCCTCCCGGCCCTCACCCCCTCCTTGGCTTTAACCTTGCGAATGTTGGTGAGATGGAT
GAATAAAGTGAATCTTTGCACTTGTGACTTCTCTCTGCTTCTTCATTTAATGGTTATTAC
TCATGGTTTCCCAGTTGCCCTAAAGTCACCGCCATTTCATCCTCCATCCCACCCTGCCCT
GCTGTCCTCCGGGAGACACCACTCCCTGAAACCCACAGGCCCCTGTCTTCACACCGCCGA
CCCCGACCACACGTGAGGGGCTTGCTTCGTGTCTCACTCCCCTCATCGAGCCCCAGAGTC
CTCCTTTAGTGTTCTTACAGTCACATACAGTTATACAGTTTGAGTCAATCCAACCTGCCC
TGCCAATTTCCCAAAACAAAGATTTTCAGAATAAAACAGCTATGAAGAAAGTCATTTATG
GAAGCATGATATACAACAACAAAACAATGCAAACAACCTAACTGAATAAGCAGAGGGAAA
TGTTCAGACACACTATGGGGCTTGGGCTTCATGGAGTATTACACCTTCATTACATTTTTA
AACTTGTATTAAGGAGCTCCTATATTACAAGGATTATACTAGAGCACTTTCCATGACCTA
ATTAATTCTCATTACACTGTGAGGTTAAAAGCATTAGTTAAAATATTGGGCAGGCTCCCT
ATAGCCAACAGTTGTTCATATTCCATAACCCAACCATCATTTAGGTGACTCAGGGTCCTT
GTCCACCAAGAACTTTGGCAAGAATGTTCAGAGCAACTTCCTTTATAAAAGTCAAAAATT
GGAAGTAACTCAAATGTCTACCAACAGTAGAATGGGCTGTTAATTGGCATATGTTTACAT
ATTAGAATGCTGTTTAATAAAGAGAATTAACAAACTACAACTATCCCTAATAACATAGGT
GACTCATAAACATGATGTTAAGCACAAGAACCCAAACACAAAAGACACACTGTGTATGTT
TTCATCCATAGGAAGTTCAAAACTAGTTAAAAATTGAATTAGAAATTGAGATGAAGTTTA
CTCTTGGCTGGGGGTGTGGAGTGAGGCGGTGCCTGGTGGGGGACAGAAAGTGGCTGCTGG
GGTCTTGGTGATGTTCTAGTCCTCACTGTGGTGTGTGCTACTCTGAAAATGTATTGAGTA
CACAATTAGGTTTTGTGCTTTCATTATACTCCAAAGTAAGTTCTCATAAACATTGCCTTA
CACGGGGTCTACAGATAAGAGAGACTAAGAGGAATGAGTAACAGATCAAGGCCACACAGC
TGGTAGGCATGGGCCTGGGATCAAACCCTGTCTGCCCAATTCTGCTCTCTTGAGCCCTAC
ACTATTCTTTCCAGCACTGGAATGCCATGCAGAACAGGGAGTAGGACATGCTACCTCCCT
AGGGTCTCCTCCTTTACCCACCTAACCAGGAGCACCCATACATAGAAACAGGATGGAAAA
GACCATCAGCAATGGAACAAGGGAGAGATTAACCTTGTTCAGTATTGTGATCCCATGTAG
GAAAGATTGTGGGAGGAGGGCTGCACACAGAGCACCGTCCCCCTTCTATGTGCCCACCGC
TCTGTGCCCCCTTATCTGCTCACCCGCCCAGCGTGCATTCACTCAGCACCCTTTTCGCCT
GCCCTCTGAAAGAGGTGCAGAAGTAACTAAACCAGCTTCCCTCCTTCAGTGACTTGGAAT
CCAGTTTTCCTCCTCTATTTCCCCCTCCTTTTCAGTGCAGGAGCCTGGAGAAATGTGATT
TGTGTTATTATAAATTTCCCACATCATTTTGTGTAAGGGAAAATATACTCAACAGTCATA
ACTGGTAAAACTGCTGTGAAAACTAAGAGAAGTAATTCATGCGAAGGTTGAGCACCAGCC
TTGTATATACTAAGAGATCCAGAAGTGTTAGTCACCGTTAGAAATAAGAAGGAGTAGCTC
AATTTGACTAGTTCCTGGTTCACTCCTTGAACATGTTCTTCAGTTATCATCTTTCAGTCC
CAAATGATTGAACTTGGAATTAACTCACATGGATTCTAGACCTGTGCCGAGAATGGCTGC
CACTCGTGCTCTAGAGCTCTGGGGATGAGGCTGTCCCTACTGTGGTGTGCTACAGGTCTA
ACAACACACCAGGTTTTGAAGACTTAGCACTATGAATATATATATATATTATATTCCAAT
AAATTTAACATACTTTCTACTTTCATTGCATGTTGAGATAGTAATCTACTTTGGATATAT
TTGGTTAAACCAAACTATTCTCAAGACAAATTTCATAGGTTTATGGTTTTTTTACAATTT
AATCAAAATATAAACATAGTCCAAACAATTAATCCATTTAAAGTGGAGAATGGCCCAAGT
GTTTGGGCCCCTGCTACCCATTTTTAAGACCAGATGTTGCTCTTGGCTTCTGGCTTTTGC
TTGGCTCAGCCCTGGCCATTGCAGCCATCTGAGGAGTAAACAGTGGATGGAAGACATCTC
CCCCCACCGTGCCCATAAAGCTCGGGATCC
```

Figure 5. Novel nucleotide sequences (SEQ ID NO:12 and SEQ ID NO: 13)
5'prime of the rabbit Cgamma gene. The sequences between SEQ ID NO: 12 and
SEQ ID NO: 13 (a gap of about 1000 nt) remain to be determined.

```
CTTCAGGGTGAAGGACGGCGGCCCGCTGAGTCTCACGCACCCCCG
GTGCAGGCAGTCCGGCCTCACCTGGAAGGTGCACTGACTGAAGACACTGCAAGGGGTGAG
AGCATTTCTCAGGAAAGAGCCCTGAGTTTAGAAGGCCAGAGAGCAGAGGGCTGAGGGCTG
CCTTGCGCTGCAACCCATGGAAACACAGGCTTAGCAGATGTTCAAGCTCCGGGAGTCCAC
ACTGGGTGAGGGCAGGCGTCCAGCCTGACATGGCCCCCACAGACTCGCCCACAGGTGACG
CCAGATGAGGACGGTCAAGGATCGGGGGGATCCTACATGCCCAGGGGCACCAAGACAGCCA
GGAGAGCACCAGAGGCCACAAGAGAGGCCTGGGACAGTCTCCCTGCTGACATCCAGAGCC
CAGGCCCCACTTGGCAGAGCTGGCTGAGAACACGTCTCTGCGGTGGAAGCTGCCCCGTCC
TGGGTGTTGCTCGGCGGGCTAAGCCGACTGACGCGGGGCGGGCCAGGCCATCGGCCCCAC
GGCCTGCAGCTTCCTCCCCAGCCCAGGCCACGTGGGCTCCTGGCTGAACTGGCCGCTCGC
TGAGCTCTCACCCCCCCACCCAGCAGCAGGCCGGGCGGTGCTGCCATGAGCTCCATTCCC
ACCACACAAGCGACAGCCCGGGCAGCGCCCCAGGCCCACGGGGCGTTTGCTGTGCGGCTC
GCACTCGCTGCTCAGGGCCAGCGCAGGGTGCAGCAGGGACTCACCAACCCGCCCCGACTC
GGCTGGCACGTTTACTGGAGGCCTCTGAGCCTGACCGTGGCAGTGGGGCCCGAGCAGGCT
CCAGGCTGCCCCCTGCACCCTGGGCTTGCCGCTCCGGGACCCCTGGTGGGCACCTTCCCA
GATGTGCTCCCACCGTGCCTCCTTGGGGCTCTGGGCTCATAGCGGTCACTCTCCGCCTTC
TCTCCTCCCAGCCCTTTCCTGCCTCCCTATGGCCCCATCTAGCTCTGCCCTNTCTAGAGC
CTCTACCTGGAAGGAATCTGCTGTTGGACCAAGACACCACCCGCAGCACAGGTGGGCGCC
TTGCACTGTGCTAGGCCCTCCCCGCACAGAAAAGGGCCCTAGGCTCTGGAGGCTGCTGCT
GNCTCTGGGGCTGGCATCGGGCGCACCCTGCACCCTGCACCCTGAGGAAACTCAGGCCTG
CCCGCTCCAGGCCTGTCCCT
```

    Gap of about 1000 nt

```
GAAGCTTTACTTGTTGGGGGCGG
GCAGGTCTAAGGGACCTGCCAGGTGTGGGGGCTGGGCTTGACTCAGCAGGAGCCTTCTAG
AAGGAAAGCTCTGGAGAAGGTGGGGGCAGAGGGCGGGAAAGGCCTGTGAGGAGGCGGGTG
GTGGGCAGGGCCACTGGGAAGGGAGGGCTGGGGGTGACACTCAGGTTGGCACTGGGGAGG
ACCTGAGGAGGCAGGTGCCAGGCACAGAGCTGAACCTGGGCAGGGCAGGGGCAGGTAACA
AGAAGGATTCTCCTTGGAGCCTGGTCCAGGGTGGTCCAGGGCGGTCCAGGGCCTGGGGTT
TGCAAGCTGGGCTGTGACAGGGCCTCTCTCCCCAGGGGCAAGCAGCAAAGCCTGGGCACA
GAGCCCAAAGCCCCCACACAGAGAAGCTCCCCAGGGCAGGGCCTGCAGGGCTTGGGGGAC
CTTCTTGGAGCAGGCAGAGGACAGAGGCATGAGATCAGCCTCCCAGAGGCTGGAATGATA
GGTCCAGCAGGAGGGGCCCACATGGGCTCTGGTTAGCAGGAGAAAACAGCCCCCAGGTCC
CCATGGCCACCACGCACCGACTGCTGGTGAAGCTTTGGGTGGCAGACGAGAGCCACATGG
CAGCTGCTCCTGTCACTGGGTGGGGCGCAGGTTCGAGGGGCGGCGGCAG
```
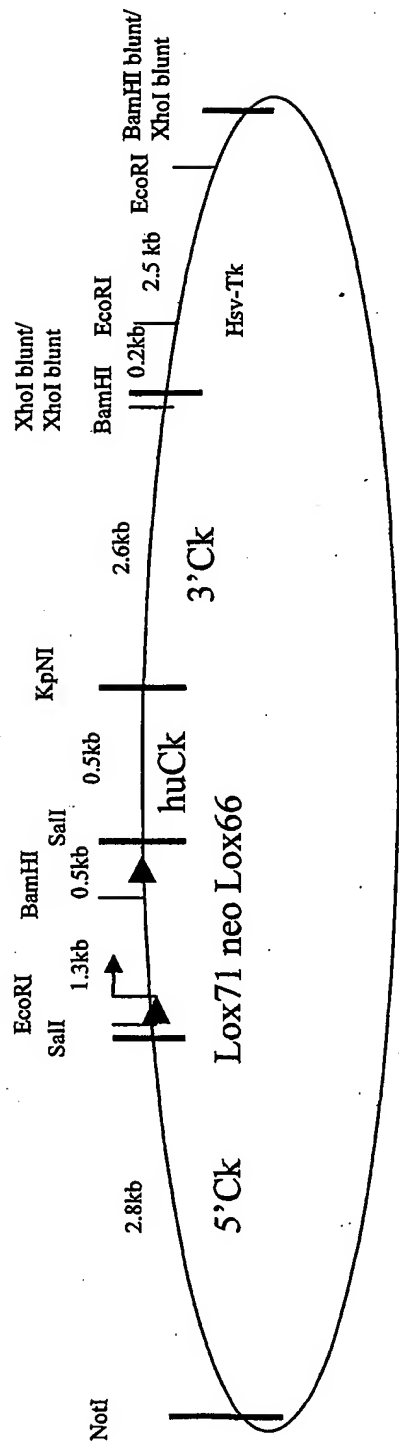
Figure 6. Comparison of human, mouse, rabbit, sheep, cow and camel sequences for the the M1 and M2 regions 3' of the Cgamma gene.

```
M1
                    1                                           46 SEQ
        camel  EPLLEEESCA EAQSGELDGL WTTISIFITL FLLSVCYSAT VTLFK. 14
    human-Ig3  .ELQLEESCA EAQDGELDGL WTTITIFITL FLLSVCYSAT VTFFK. 15
  human-Ig3/2  .ELQLEESCA EAQDGELDGL WTTITILITL FLLSVCYSAT VTFFK. 16
    human-Ig1  .ELQLEESCA EAQDGELDGL WTTITIFITL FLLSVCYSAT VTFFK. 17
    mouse-Ig1  .GLQLDETCA EAQDGELDGL WTTITIFISL FLLSVCYSAA VTLFK. 18
   mouse-Ig2a  .GLDLDDVCA EAQDGELDGL WTTITIFISL FLLSVCYSAS VTLFK. 19
   mouse-mRNA  PGLQLDETCA EAQDGELDGL WTTITIFISL FLLSVCYSAA VTLFK. 20
    mouse-Ig3  .ELELNETCA EAQDGELDGL WTTITIFISL FLLSVCYSAS VTLFK. 21
  mouse-Ig3/2  .ELELNGTCA EAQDGELDGL WTTITIFISL FLLSVCYSAS VTLFK. 22
 sheep-clone11  .LLLEEESCA DAQDGELDGL WTTISIFITP FLLSVCYSAT VTLFK. 23
  sheep-clone1  .LLLEEESCA DAQDGELDGL WTTISIFITL FLLSVCYSAT VTLFK. 24
   cow-clone11  .LLLEEEICA DDLDGELDGL .......... .......... ...... 25
  cow-clone3/5  .LLLEEEICA DAQDGELDGL .......... .......... ...... 26
       rabbit  ..LQLDESCA EAQDGELDGL WTTITIFISL FLLSVCYSAT VTLFK. 27
```
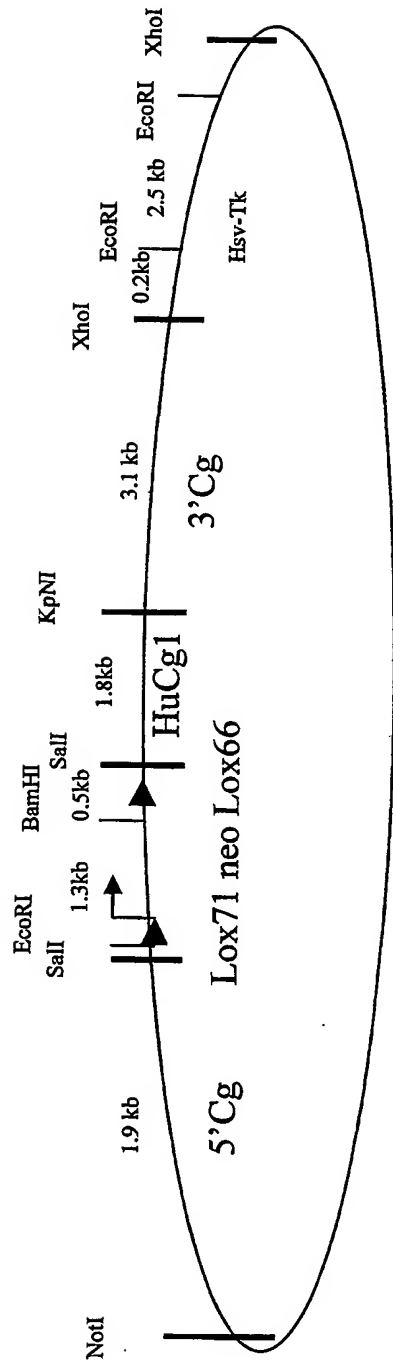
```
M2
                    1                  27                    SEQ
        camel  VKWIFSSVVE LKRTIVPDYR NMIGQGS                  28
    human-Ig3  VKWIFSSVVD LKQTIIPDYR NMIGQGA                  29
  human-Ig3/2  VKWIFSSVVD LKQTIIPDYR NMIGQGA                  30
    human-Ig1  VKWIFSSVVD LKQTIIPDYR NMIGQGA                  31
    mouse-Ig1  VKWIFSSVVE LKQTLVPEYK NMIGQAP                  32
   mouse-Ig2a  VKWIFSSVVE LKQTISPDYR NMIGQGA                  33
   mouse-mRNA  VKWIFSSVVE LKQTLVPEYK NMIGQAP                  34
    mouse-Ig3  VKWIFSSVVQ VKQTAIPDYR NMIGQGA                  35
  mouse-Ig3/2  VKWIFSSVVQ VKQTAIPDYR NMIGQGA                  36
       rabbit  VKWIFSSVVE LKHTIAPDYR NMMGQGA                  37
 sheep-clone1/11  VKWIFSSV.. ..........                      38
```

Figure 7a: DNA construct for the replacement of rabbit Cκ with human Cκ. A 0.5 kb fragment containing a DNA sequence encoding human Cκ is flanked by sequences from the rabbit Cκ1 gene. The upstream sequence (5'Cκ) is 2.8 kb, the downstream sequence (3'Cκ) is 2.6 kb. The vector also contains a lox-neo cassette for positive selection and a Hsv-Tk cassette for negative selection.

Figure 7b: DNA construct for the replacement of rabbit Cγ with human Cγ1. A 1.8 kb fragment containing a DNA sequence encoding human Cγ1 is flanked by sequences from the rabbit Cγ gene. The upstream sequence (5'Cγ) is 1.9 kb, the downstream sequence (3'Cγ) is 3.1 kb. The vector also contains a lox-neo casette for positive selection and a Hsv-Tk cassette for negative selection. The figure is not up to scale.

Figure 8. DNA fragment (SEQ ID NO: 51) containing a human immunoglobulin heavy chain Cγ1 gene segment flanked by 50 nucleotides derived from the rabbit heavy chain immunoglobulin gene. Flanking sequences derived from rabbit immunoglobulin DNA sequences are underlined.

```
                          tgacctacct accctgccaa ggtcaggggt cctccaaggc
aagggatcac atggcaccac ctctcttgca gcctccacca agggcccatc ggtcttcccc
ctggcaccct cctccaagag cacctctggg ggcacagcgg ccctgggctg cctggtcaag
gactacttcc ccgaaccggt gacggtgtcg tggaactcag gcgccctgac cagcggcgtg
cacaccttcc cggctgtcct acagtcctca ggactctact ccctcagcag cgtggtgacc
gtgccctcca gcagcttggg cacccagacc tacatctgca acgtgaatca caagcccagc
aacaccaagg tggacaagaa agttggtgag aggccagcac agggagggag ggtgtctgct
ggaagccagg ctcagcgctc ctgcctggac gcatcccggc tatgcagccc cagtccaggg
cagcaaggca ggccccgtct gcctcttcac ccggaggcct ctgcccgccc cactcatgct
cagggagagg gtcttctggc ttttttcccca ggctctgggc aggcacaggc taggtgcccc
taacccaggc cctgcacaca aaggggcagg tgctgggctc agacctgcca agagccatat
ccgggaggac cctgcccctg acctaagccc accccaaagg ccaaactctc cactccctca
gctcggacac cttctctcct cccagattcc agtaactccc aatcttctct ctgcagagcc
caaatcttgt gacaaaactc acacatgccc accgtgccca ggtaagccag cccaggcctc
gccctccagc tcaaggcggg acaggtgccc tagagtagcc tgcatccagg acaggcccc
agccgggtgc tgacacgtcc acctccatct cttcctcagc acctgaactc ctggggggac
cgtcagtctt cctcttcccc ccaaaaccca aggacaccct catgatctcc cggacccctg
aggtcacatg cgtggtggtg gacgtgagcc acgaagaccc tgaggtcaag ttcaactggt
acgtggacgg cgtggaggtg cataatgcca agacaaagcc gcgggaggag cagtacaaca
gcacgtaccg tgtggtcagc gtcctcaccg tcctgcacca ggactggctg aatggcaagg
agtacaagtg caaggtctcc aacaaagccc tcccagcccc catcgagaaa accatctcca
aagccaaagg tgggacccgt ggggtgcgag ggccacatgg acagaggccg gctcggccca
ccctctgccc tgagagtgac cgctgtacca acctctgtcc ctacagggca gccccgagaa
ccacaggtgt acaccctgcc cccatcccgg gatgagctga ccaagaacca ggtcagcctg
acctgcctgg tcaaaggctt ctatcccagc gacatcgccg tggagtggga gagcaatggg
cagccggaga caaactacaa gaccacgcct cccgtgctgg actccgacgg ctccttcttc
ctctacagca agctcaccgt ggacaagagc aggtggcagc aggggaacgt cttctcatgc
tccgtgatgc atgaggctct gcacaaccac tacacgcaga agagcctctc cctgtctccg
ggtaaatgag cgctgtgccg gcgagctgcc cctctccctc cccccacgc cgcagctgt.
```

Figure 9. The DNA fragment (SEQ ID NO: 52) containing a VH gene segment with more than 80% sequence identity with rabbit VH elements and encoding a human VH element polypeptide sequence. Flanking sequences derived from rabbit immunoglobulin DNA sequences are underlined.

```
                tgagtgacag tgtcctgacc atgtcgtctg tgtttgcagg tgtccagtgt
      gaggtgcagc tgttggagtc cggggggaggt ctcgtccagc caggggggac cctgagactc
      acctgcgcag tctctggatt caccttcagt agctatgcaa tgagctgggt ccgccaggct
      ccagggaagg ggctggaatg ggtcggagcc attagtggta gtggtagcac atactacgcg
      gacagcgtga aaggccgatt caccatctcc agagacaact ccaagaacac gctgtatctg
      caaatgaaca gtctgagagc cgaggacacg gccgcctatt actgtgcgaa agacacagtg
      aggggccctc aggctgagcc cagacacaaa cctccctgca
```

Figure 10. DNA fragment (SEQ ID NO: 53) containing a human immunoglobulin light chain Cк gene segment flanked by 50 nucleotides derived from the rabbit light chain immunoglobulin Kappal gene. Flanking sequences derived from rabbit immunoglobulin DNA sequences are underlined.

```
                                               .catccacat ggcacccagg
      ggagatgtcc actggtacct aagcctcgcc atcctgtttg cttctttcct caggaactgt
      ggctgcacca tctgtcttca tcttcccgcc atctgatgag cagttgaaat ctggaactgc
      ctctgttgtg tgcctgctga ataacttcta tcccagagag gccaaagtac agtggaaggt
      ggataacgcc ctccaatcgg gtaactccca ggagagtgtc acagagcagg acagcaagga
      cagcacctac agcctcagca gcaccctgac gctgagcaaa gcagactacg agaaacacaa
      agtctacgcc tgcgaagtca cccatcaggg cctgagctcg cccgtcacaa agagcttcaa
      caggggagag tgttagagcg agacgcctgc caggcaccg ccagcgaccc tgaggcccag
      cctcgc.
```

Figure 11. DNA fragment (SEQ ID NO: 54) containing a Vк gene segment with more than 80% sequence identity with rabbit Vк elements and encoding a human Vк element polypeptide sequence. Flanking sequences derived from rabbit immunoglobulin DNA sequences are underlined.

```
                                            ..ggcaggct gctcccaccc
      catgcaggag gcagtaccag gcaggaccca gcatggacat gagggtccct gctcagctcc
      tgggactcct gctgctctgg ctcccaggta aggaggggaaa caacaaaaat tttattcagc
      cagtgtagcc actaatgcct ggcacttcag gaaattcttc ttagaacatt actaatcatg
      tggatatgtg tttttatgtt cctaatatca gataccagat gttacatcca gatgacccag
      tctccatcct ctctgtctgc atctgtggga gacagagtca ccatcacttg ccgagccagt
      cagggcatta gcaattactt agcctggtat cagcagaaac cagggaaggt tcccaagctc
      ctgatttatg ctgcatccac tttgcaatct ggggtcccat cgcggttcag tggcagtgga
      tctgggacag atttcactct taccatcagc agcctgcagc ctgaagatgt tgccacctat
      tactgtcaaa agtacaacag tgcccctcca cttttcggcg gagggaccaa ggtggagatc
      aaacgtaagt gcactttcct aatgttcctc accgtttctg cctgatttgt ttgctttttc
      cattttttcgctat..
```

Figure 12. DNA fragment (SEQ ID NO: 57) containing a gene encoding human immunoglobulin light chain constant region Clambda2 flanked by 50 nucleotides derived from the chicken light chain gene. The DNA sequence of chicken origin is underlined.

```
catacacag ccatacatac gcgtgtggcc gctctgcctc tctcttgcag gtcagcccaa
ggctgccccc tccgtcactc tgttcccgcc ctcctctgag gagcttcaag ccaacaaggc
cacactggtg tgtctcataa gtgacttcta cccgggagcc gtgacagtgg cttggaaagc
agatagcagc cccgtcaagg cgggagtgga gaccaccaca ccctccaaac aaagcaacaa
caagtacgcg gccagcagct atctgagcct gacgcctgag cagtggaagt cccacagaag
ctacagctgc caggtcacgc atgaagggag caccgtggag aagacagtgg cccctacaga
atgttcatag tagtcccact ggggatgcaa tgtgaggaca gtggttcctc accctccctg
```

Figure 13. Modification of the chicken light chain locus using the ET system.
A chicken genomic BAC clone with the full length light chain locus was modified by homologous recombination. In a first step Cλ was deleted by insertion of a selection cassette which was in a second homologous recombination step exchanged against the human Cλ gene. The homology stretch was 50bp.

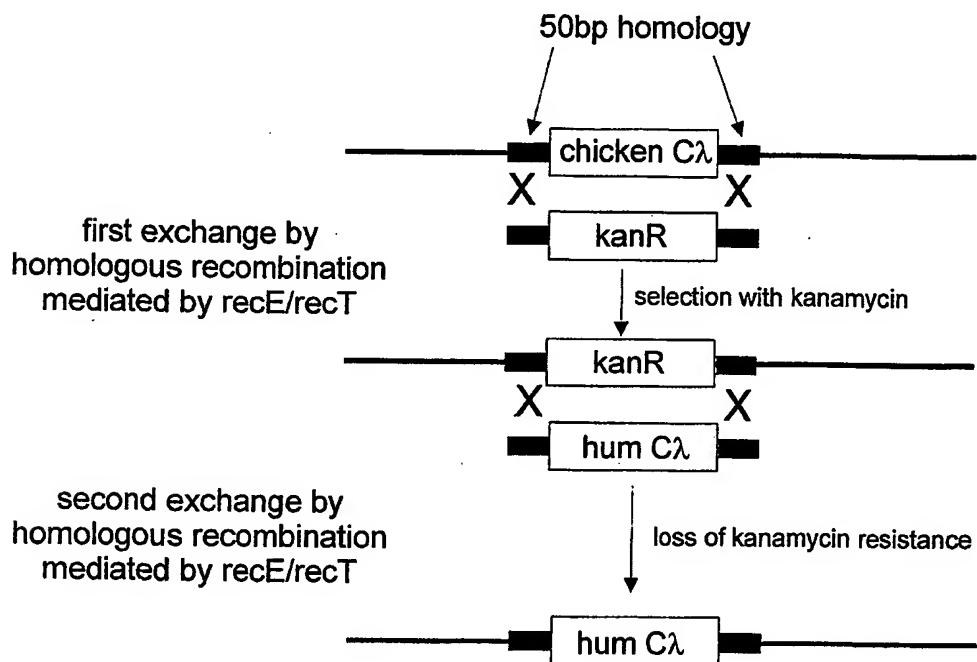Figure 14. DNA fragment (SEQ ID NO: 58) containing a VJ gene segment with 80% sequence identity with chicken V gene segments and encoding a human VJ immunoglobulin polypeptide. Flanking sequences derived form chicken immunolgobulin DNA sequences are underlined.

<u>.ttgccgttt tctcccctct</u> ctcctctccc tctccaggtt ccctggtgca gtcagtgctg actcagccgc
cctcggtgtc agcagccccg ggacaagaag tcacgatctc ctgctccggg tctagtagca acattggcga
taatttcgtc tcttggtacC agcagctgcc tggcactgcc cctaagcttc tgatctatga taacaacAag
agaccctcgg gcatccctga ccgattctcc ggttccaaat ccggcacctc agccacatta ggcatcactg
ggctccaaac cggcgacgag gctgactatt actgtgggac ttgggacagc agcctttctg ttggtatgtt
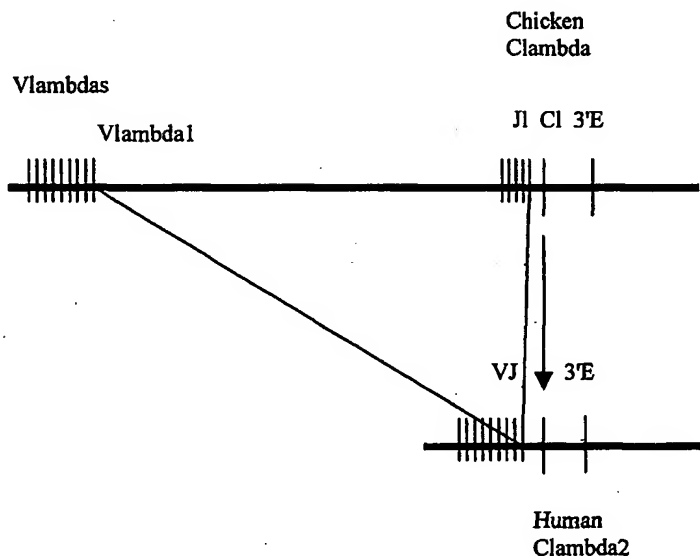tgggggcggg acacgcg<u>tga ccgtcctagg tgagtcgctg acctcgtctc ggtctttctt ccccccat</u>...

Figure 15. Humanized chicken light chain locus.